SURVEILLANCE AND GENETIC CHARACTERIZATION OF SEVERE ACUTE RESPIRATORY CORONAVIRUS TYPE 2 (SARS-CoV-2) INFECTIONS IN DOMESTIC ANIMALS AND WILDLIFE IN THAILAND

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การเฝ้าระวังการติดเชื้อและลักษณะทางพันธุกรรมของเชื้อไวรัสโคโรนาสายพันธุ์กลุ่มโรคทางเดินหายใจเฉียบพลันรุนแรงชนิดที่ 2 (SARS-CoV-2)ในสัตว์เลี้ยงและสัตว์ป่าในประเทศไทย

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วลีมาศ ใจรัก : การเฝ้าระวังการติดเชื้อและลักษณะทางพันธุกรรมของเชื้อไวรัสโคโรนาสายพันธุ์กลุ่มโรคทางเดินหายใจเฉียบพลันรุนแรงชนิดที่ 2 (SARS-CoV-2)ในสัตว์เลี้ยงและสัตว์ป่าในประเทศไทย. (SURVEILLANCE AND GENETIC CHARACTERIZATION OF SEVERE ACUTE RESPIRATORY CORONAVIRUS TYPE 2 (SARS-CoV-2) INFECTIONS IN DOMESTIC ANIMALS AND WILDLIFE IN THAILAND) อ.ที่ปรึกษาหลัก : ศ. น.สพ. ดร.อลงกรณ อมรศิลป์D.V.M. Ph.D.

เชื้อไวรัสโคโรนาสายพันธุ์กลุ่มโรคทางเดินหายใจเฉียบพลันรุนแรงชนิดที่ 2 (SARS-CoV-2) รายงานพบเป็นครั้งแรกในเดือนธันวาคม พ.ศ. 2562 เป็นสาเหตุของการระบาดใหญ่ของโลก โดยมีผลกระทบไปทั่วโลกทั้งด้านสาธารณสุข เศรษฐกิจ และสังคม ซึ่งวิทยานิพนธ์เรื่อง “การเฝ้าระวังและลักษณะทางพันธุกรรมของการติดเชื้อไวรัสทางเดินหายใจเฉียบพลันชนิดที่ 2 (SARS-CoV-2) ในสัตว์เลี้ยงและสัตว์ป่าในประเทศไทย” นี้ มีวัตถุประสงค์เพื่อศึกษาอุบัติการณ์การแพร่เชื้อไวรัสระหว่างคนและสัตว์และลักษณะของไวรัสที่พบ โดยวิทยานิพนธ์นี้ประกอบด้วยการศึกษาจำนวน 4 เรื่อง ในการเฝ้าระวังเชื้อไวรัส SARS-CoV-2 ในสัตว์เลี้ยงและสัตว์ป่าในประเทศไทย ระหว่างปี พ.ศ. 2560-2566 โดยงานวิจัยเรื่องที่ 1 (บทที่ 2) เป็นการสำรวจเชื้อไวรัส SARS-CoV-2 ในสุนัขและแมว ในจังหวัดสมุทรสาครซึ่งเป็นพื้นที่เสี่ยงสูงต่อโรคโควิด-19 ระหว่างการระบาดระลอกที่สองของโรคโควิด-19 ในประเทศไทยในช่วงเดือนกุมภาพันธ์ พ.ศ. 2564 แม้การศึกษานี้จะตรวจไม่พบสารพันธุกรรมของไวรัสในตัวอย่างจากสุนัขและแมวที่ศึกษา แต่อย่างไรก็ตามพบว่า 3.14% (5/159) ของกลุ่มศึกษาพบผลบวกต่อการตรวจภูมิคุ้มกันต่อเชื้อไวรัส SARS-CoV-2 ชนิด anti-N-IgG ซึ่งบ่งชี้ถึงโอกาสสัมผัสโรคของสัตว์เหล่านี้ในพื้นที่เสี่ยงสูง การศึกษาเรื่องที่ 2 (บทที่ 3) เป็นการศึกษาเฝ้าระวังเชิงรุกต่อเชื้อไวรัส SARS-CoV-2 ในสุนัขและแมวจากบ้านที่พบ COVID-19 ในคน ในช่วงการระบาดในระลอกที่ 3 ของประเทศไทยคือเดือนเมษายนถึงพฤษภาคม 2564 การศึกษานี้ได้ยืนยันการติดเชื้อไวรัส SARS-CoV-2 ในสุนัขและแมว โดยตรวจพบสารพันธุกรรมและภูมิคุ้มกันต่อเชื้อไวรัส SARS-CoV-2 ในสัตว์จำนวน 4 ตัวจากทั้งหมด 44 ตัว และการวิเคราะห์แผนภูมิวิวัฒนาการ พบว่าไวรัสที่พบจัดอยู่ในสายพันธุ์ Alpha (B.1.1.7) การศึกษาที่ 3 (บทที่ 4) เป็นการสำรวจเชิงภาคตัดขวาง (cross-sectional survey) ในพื้นที่กรุงเทพมหานครและใกล้เคียงในช่วงที่มีการระบาดของโรคโควิด-19 ระลอกที่ 4 ของประเทศไทย ซึ่งสามารถยืนยันการติดเชื้อ SARS-CoV-2 ในสุนัขและแมวจากบ้านที่พบ COVID-19 ในคน โดยเชื้อไวรัส SARS-CoV-2 ที่พบในสัตว์จัดอยู่ในสายพันธุ์ Delta (B.1.617.2) การศึกษานี้สร้างความตระหนักเกี่ยวกับการแพร่เชื้อไวรัสสายพันธุ์ที่น่ากังวลจากคนสู่สัตว์เลี้ยง การศึกษาเรื่องที่ 4 (บทที่ 5) เป็นการศึกษาเฝ้าระวังเชื้อไวรัส SARS-CoV-2 ในสัตว์ป่า ในสถานที่เพาะเลี้ยงและในถิ่นอาศัยตามธรรมชาติ ในช่วงก่อนและหลังโควิด-19 (พ.ศ. 2560 ถึง พ.ศ. 2566) ผลการศึกษาพบสิงโตจำนวน 7 ตัวในสวนสัตว์แห่งหนึ่งได้ตรวจพบภูมิคุ้มกันต่อเชื้อไวรัส SARS-CoV-2 จากนั้นจึงได้ทำการศึกษาย้อนหลัง (retrospective study) และได้ตรวจพบไวรัสในเสือโคร่งในสวนสัตว์ดังกล่าวเช่นกัน การศึกษานี้ชี้ให้เห็นถึงความเป็นไปได้ในการเกิดการแพร่เชื้อไวรัสจากคนสู่สัตว์ในเสือและสิงโต การค้นพบนี้เน้นย้ำถึงความสำคัญของการเฝ้าระวังโรคทั้งเชิงรุกและเชิงรับ โดยเฉพาะในสัตว์ที่ประวัติสัมผัสคนเลี้ยงที่เป็นโควิด-19 โดยสรุปแล้ววิทยานิพนธ์ฉบับนี้สามารถเสริมสร้างความตระหนักรู้ของสาธารณชน การสื่อสารความเสี่ยง และความเข้าใจเกี่ยวกับลักษณะและปัจจัยที่เกี่ยวข้องของการติดเชื้อ SARS-CoV-2 ในสัตว์เลี้ยงและสัตว์ป่า

สาขาวิชา	สัตวแพทยสาธารณสุข	ลายมือชื่อนิสิต .....
ปีการศึกษา	2566	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORD: domestic animals, severe acute respiratory coronavirus type 2 (SARS-CoV-2), Thailand, wildlife

Waleemas Jairak : SURVEILLANCE AND GENETIC CHARACTERIZATION OF SEVERE ACUTE RESPIRATORY CORONAVIRUS TYPE 2 (SARS-CoV-2) INFECTIONS IN DOMESTIC ANIMALS AND WILDLIFE IN THAILAND. Advisor: Prof. Dr. ALONGKORN AMONSIN, D.V.M. Ph.D.

SARS-CoV-2 caused a pandemic outbreak since December 2019 with global implications for public health, economy, and society. This dissertation entitled “Surveillance and genetic characterization of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infections in domestic animals and wildlife in Thailand” investigates the transmission incidence and characterization of the virus between humans and animals. This thesis comprises 4 studies of the surveillance of SARS-CoV-2 in animals and wildlife species in Thailand from 2017 to 2023. The first investigation (Chapter 2) involved a survey of SARS-CoV-2 in domestic dogs and cats in Samut Sakhon province during the second wave of the COVID-19 outbreak in Thailand in February 2021. Although no viral RNA was detected in the swab samples, 3.14% (5/159) of studied animals tested positive for anti-N-IgG antibody, indicating possible SARS-CoV-2 exposure in high-risk areas. During the third wave of the pandemic, from April to May 2021, the second investigation (Chapter 3) provided active surveillance of SARS-CoV-2 among dogs and cats from COVID-19 households. The detection of SARS-CoV-2 RNA and antibodies in four of the 44 animals confirmed SARS-CoV-2 infection. According to phylogenetic analyses, the viral strains belonged to the Alpha VOCs (B.1.1.7 lineage). The third study (chapter 4) described a cross-sectional survey conducted in Bangkok and vicinities during Thailand’s 4th wave of the COVID-19 outbreak. SARS-CoV-2 infections were discovered in a dog and a cat from COVID-19 positive households. The viral genomes were classified as Delta variant (B.1.617.2 lineage). This study raised awareness of the spillover of variants of concern in domestic animals. The fourth study (Chapter 5), expanded the surveillance of wildlife species in captive and free-ranging habitats during pre and post-COVID-19 (2017 to 2023). Seven lions of a zoo tested positive for SARS-CoV-2 immunity, and a retrospective investigation in the same zoo confirmed the virus infection in a tiger. Human-to-animal transmission has been proposed in the tiger and lions. The finding emphasized the importance of monitoring COVID-19 exposure history in animal species through passive and active surveillance. The summary information of this dissertation contributes to public awareness, risk communication, and the understanding of SARS-CoV-2 dynamics in domestic animals and wildlife populations.

Field of Study:	Veterinary Public Health	Student's Signature .....
Academic Year:	2023	Advisor's Signature .....

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LIST OF ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
bp	Base pair(s)
CDC	Centers for Disease Control and Prevention
CCoV	Canine coronavirus
CECoV	Canine enteric coronavirus
CRCoV	Canine respiratory coronavirus
BatCoV	Bat coronavirus
cDNA	Complementary deoxyribonucleic acid
COVID-19	Coronavirus disease of 2019
Ct	Cycle threshold
CUEIDAS	The Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals
dl	Deciliter(s)
dpi	Day-post-inoculation
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DNP	The Department of National Parks, Wildlife and Plant Conservation
E	Envelope protein
ELISA	Enzyme-linked immunosorbent assay
et al.	Et alibi, and others
FAM	Fluorescein amidites
FCoV	Feline coronavirus
FIP	Feline infectious peritonitis
fl	Femtoliter(s)
g	Gram(s)
GAPDH	The glyceraldehyde-3-phosphate dehydrogenase protein

GISAID	The Global Initiative on Sharing All Influenza Data
GFP	Green Fluorescent Protein
hACE2	Human *angiotensin-converting enzyme 2*
HCl	Hydrogen Chloride
HCoV	Human coronavirus
HRP	Horseradish peroxidase
HR2	Heptapeptide repeat sequence 2
IACUC	The Institutional Animal Care and Use Committee
IBV	Infectious bronchitis virus
IC50	Inhibitory concentration at 50%
IgG	Immunoglobulin G
Kb	Kilobase pair(s)
L	Liter(s)
M	Membrane protein
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
mg	Miligram(s)
ml	Milliliter(s)
mmol	Millimole(s)
MgSO4	Magnesium Sulfate
mRNA	Messenger ribonucleic acid
N	Nucleocapsid protein
nCoV	Novel coronavirus
nm	Nanometer(s)
nM	Nanomolar(s)
NSP	Non-structural protein
nt	Nucleotide
NTD	The N-terminal-domain
min	Minute(s)

mM	Millimolar
OD	Optical density
OIE	The Office International des Epizooties
ORFs	Open reading frames
PANGOLIN	The Phylogenetic Assignment of Named Global Outbreak Lineages tool
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
pg	Picogram(s)
PEAV	Porcine enteric alphacoronavirus
PEDV	Porcine epidemic diarrhea virus
pH	Potential of hydrogen
PCR	Polymerase chain reaction
pVNT	Pseudotyped virus neutralization
RBD	Receptor binding domain
RBM	The receptor binding motif
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcriptase-polymerase chain reaction
S	Spike protein
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
sVNT	Surrogate virus neutralization test
S/P ratio	Scotopic/photopic ratio
TCID50	The 50% Tissue Culture *Infectious* Dose
TGEV	Transmissible gastroenteritis coronavirus
TMB	Tetramethylbenzidine
VOCs	Variant of Concerns

VOIs	Variants of interests
VNT	Virus neutralization
VUM	Variant under monitoring
WHO	The World Health Organization
WOAH	World Organisation for Animal Health
U	Unit(s)
ZPOT	The Zoological Park Organization of Thailand
°C	Degree Celsius
μl	Microliter(s)
μM	Micromolar

CHAPTER 1

Introduction

1.1 Importance and Rationale

Coronaviruses are classified into four genera including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. *Alphacoronavirus* and *Betacoronavirus* can infect and cause diseases in mammals, while *Gammacoronavirus* and *Deltacoronavirus* mostly found in birds and occasionally in mammals. In human, members of *Alphacoronavirus* and *Betacoronavirus* cause mild respiratory tract infections. Notably, some members of *Betacoronavirus* are zoonotic pathogens and can cause severe respiratory syndrome (Woo *et al.*, 2012; Ar Gouilh *et al.*, 2018). In the last two decades, *Betacoronaviruses* are responsible for three major severe respiratory diseases in humans including severe acute respiratory syndrome coronavirus (SARS-CoV) causing SARS in 2003 with 774 deaths from 8,096 human cases (Cheng *et al.*, 2007), Middle East respiratory syndrome coronavirus (MERS-CoV) causing MERS in 2012 with 858 deaths from 2,499 confirmed human cases (Memish *et al.*, 2020), and recently severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing pandemic outbreak of COVID-19. As of April 2022, more than 6.2 million deaths from 511 million COVID-19 human cases (WHO, 2022a).

The viruses, SARS-CoV, MERS-CoV and SARS CoV-2, have been speculated to originate from animals and thus are considered as zoonotic pathogens. For example, in SARS-CoV causing SARS, palm civets and raccoon dogs in wildlife meat market were suspected as the source of human infection (Cheng *et al.*, 2007). In MERS-CoV causing MERS, dromedary camels were identified as reservoirs for the viruses (Memish *et al.*, 2020). In SARS-CoV-2 causing COVID-19, pangolin was suspected as intermediate host for SARS-CoV-2. Bat coronavirus RaTG13, isolated in 2013 in Yunnan, China, and pangolin coronavirus, isolated in 2019 in Guangdong, China, posed high nucleotide similarity to SARS-CoV-2 (88.9%-96.2%) suggesting bat and pangolin as animal origin or intermediate host of SARS-CoV-2 (Zhang *et al.*, 2020b;

Zhou *et al.*, 2020). It is noted that several publications reported the origin of SARS-CoV, MERS-CoV and SARS-CoV-2, which were originated from bat viruses (Lau *et al.*, 2005; Memish *et al.*, 2013; Zhou *et al.*, 2020)

Outbreak of SARS-CoV-2 was first reported in December 2019 in Wuhan city of China with epidemiologically linked to the seafood market and live animal market (WHO, 2020b). In the early stage of the outbreak in China, there were evidences suggesting zoonotic event of SARS-CoV-2 with limited human-to-human transmission (Riou and Althaus, 2020). Clinical signs of SARS-CoV-2 infection in human including fever, fatigue, dry cough, dyspnea and death (Wang *et al.*, 2020a; Zhou *et al.*, 2020). The studies of SARS-Cov-2 in Wuhan in February 2020 demonstrated the case fatality were 2.3% from total 40,261 confirmed cases with 909 deaths comparing with fatality rate 9.6% of SARS and 34.4% of MERS (She *et al.*, 2020). After 3 months of SARS-CoV-2 emerging, the virus spread to 114 countries worldwide and WHO announced this diseases as pandemic outbreak on March 11, 2020 (WHO, 2020a).

SARS-CoV-2 has high mutation rate and able to generate new SARS-CoV-2 variants due to high rate of viral replication, viral recombination and point mutation from selective pressure (Gribble *et al.*, 2021; Otto *et al.*, 2021). Up to date, the Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) and the NextStrain system are commonly used for SARS-CoV-2 variant classification (Hodcroft, 2021; Rambaut *et al.*, 2021). Up to date, WHO has announced five variants as the variant of concern (VOC) that have impact on disease transmission and human health including the Alpha variant (B.1.1.7), Beta variant (B.1.351), Gamma variant (P.1), Delta variant (B.1.617.2), and Omicron variant (B.1.1.529) (WHO, 2022c).

Since 2019, SARS-CoV-2 causes pandemic outbreak in human population and occasionally spillover to infect domestic animals and wildlife (Sit *et al.*, 2020; Hale *et al.*, 2022). SARS-CoV-2 infection have been reported in at least 23 animal species including domestics and wildlife from 35 countries worldwide (OIE, 2022). Most of SARS-CoV-2 infection in domestic animal cases were occurred due to close

contact of animals with COVID-19 human cases. Some studies reported that there were some evidences indicating animals potentially transmitted the SARS-CoV-2 back to humans such as minks in the Netherlands and Denmark, and hamsters in Hong Kong (Hammer *et al.*, 2021; Oude Munnink *et al.*, 2021; Yen *et al.*, 2022). Moreover, there are few reports of animal-to-animal transmission of SARS-CoV-2 that probably circulating in animal population such as white-tailed deer (*Odocoileus virginianus)*, in the USA and Canada (Hale *et al.*, 2022). The rapidly transmissible Omicron variant (B.1.1.529) of SARS-CoV-2 suggested that the virus can be introduced from humans to more varieties of animal species and the viruses may reversely spill back to humans (Khairnar *et al.*, 2022).

In Thailand, as of May 2022, there have been 4.3 million confirmed human cases with more than 29,000 deaths. Up to date, at least 5th wave of SARS-CoV-2 outbreaks have been reported and the recent outbreak is predominantly caused by the Omicron variant (WHO, 2022d). Since domestic animals and wildlife can be infected with SARS-CoV-2 due to their potential human-animal interface with owners or caretakers. The surveillance of SARS-CoV-2 in domestic animals and wildlife species will provide comprehensive epidemiological information of COVID-19 in animals. The results this proposal can provide the status and genetic characteristics of SARS-CoV-2 in animals in Thailand for risk communication, mitigation and management as well as diseases prevention and control.

1.2 Research questions

Due to limited information of SARS-CoV-2 in animals in Thailand, the research questions of this study were:

1. Are there any evidence or occurrence of SARS-CoV-2 infection in domestic animals and wildlife in Thailand?
2. What are the genetic characteristics and variants of SARS-CoV-2 infected in domestic animals and wildlife in Thailand?

1.3 Objectives of study

Due to the status and characteristics of SARS-CoV-2 in animals were important for risk communication, mitigation and management to public. The objectives of this study were:

1. To determine the occurrence of SARS-CoV-2 in domestic animals and wildlife in Thailand
2. To determine the genetic characteristics and variants of SARS-CoV-2 recovered from domestic animals and wildlife in Thailand.

1.4 Literature Review

1.4.1 Coronavirus

Coronavirus is an enveloped, non-segmented single strand RNA virus with 25-32 kb in length and 118-136 nm in diameter. Family *Coronaviridae* contains two subfamilies including the *Coronavirinae* and the *Torovirinae,* which *Coronavirinae* contains the coronaviruses. Coronavirus genome contains structural proteins with spike protein (S), envelope protein (E), membrane protein (M) and nucleocapsid protein (N), and non-structural proteins (NSP) with 7-10 open reading frames (ORFs) (Payne, 2017). Up to date, the *Coronavirinae* is divided into four genera including *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (Fehr and Perlman, 2015).

Alphacoronaviruses can infect human and cause mild respiratory symptoms and endemic circulation in humans such as HCoV 299E and HCoV NL63 (Corman *et al.*, 2018). In animals, *Alphacoronaviruses* cause serious respiratory and enteric diseases in livestock and domestic animals such as Transmissible gastroenteritis coronavirus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine enteric alphacoronavirus (PEAV), canine enteric coronavirus (CCoV) and feline infectious peritonitis (FIP) (Ghai *et al.*, 2021).

Betacoronaviruses can infect human and cause asymptomatic or mild respiratory symptoms as well as severe respiratory infection or even death (To *et al.*,

2013). *Betacoronaviruses* consist of five subgenera including *Embecovirus*, *Sarbecovirus*, *Merbecovirus*, *Nobecovirus* and *Hibecovirus*. The human coronaviruses including HCoV HKU1, HCoV OC43 and animal coronaviruses such as bovine coronaviruses, equine coronaviruses, porcine coronaviruses and murine coronaviruses, belong to *Embecoviruses*. SARS-CoV, SAR-CoV-2 and animal SAR-like CoV belong to *Sarbecovirus*. MER-CoV and some bat coronaviruses belong to *Merbecovirus*. Bat coronavirus isolated from *Rousettus* and *Hipposideros* bats belong to *Nobecovirus* and *Hibecovirus* respectively (Llanes *et al.*, 2020). Notably, *Betacoronavorisuses* are the cause of three major pandemic outbreaks in humans in the last 20 years including SARS-CoV in 2003, MERS-CoV in 2012 and SARS-CoV-2 since 2019 up to present (Hui *et al.*, 2022).

Gammacoronaviruses are mainly found in avian species (Decaro, 2011). *Gammacoronaviruses* have been isolated from avian species including domestic poultry and wild birds, as well as in mammals such as beluga whales (Mihindukulasuriya *et al.*, 2008; Decaro, 2011). Among various *Gammacoronavirus* species, the infectious bronchitis virus (IBV) is the most important pathogen affecting animal health and economic impact in poultry industry (Jackwood *et al.*, 2012).

Deltacoronaviruses are found in mammals and avian species such as bulbuls, thrushs, munias, white-eyes, sparrows, robins, herons, wigeons, moorhens, falcons, bustards, pigeons and quails, as well as in mammals such as pigs (Woo *et al.*, 2012). *Deltacoronaviruses* have been reported in many wild bird species without any clinical signs. In contrast, *Deltacoronaviruses* have been reported to cause severe diarrhea in pigs in China, and the United States (Woo *et al.*, 2012; Wang *et al.*, 2014; Dong *et al.*, 2015).

1.4.2 The severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2)

SARS-CoV-2 was firstly reported as emerging virus in Wuhan, Hubei province of China in December 2019. SARS-CoV-2 infection was first linked to the visitor at the

seafood and wild animal market such as workers and frequent visitors (Bagcchi, 2020). In January 2020, the Chinese research team identified the causative agent of this outbreak as a novel coronavirus belonging to *Sarbecovirus* of *Betacoronavirus* genus (Zhu *et al.*, 2020). SARS-CoV-2 infection causes fever, fatigue and dry cough, and in severe cases causing dyspnea, pneumonia and death (Wang *et al.*, 2020a; Zhou *et al.*, 2020). SARS-CoV-2 can spread via direct transmission by the inhalation of droplets and aerosol particles, and via indirect transmission by contact the contaminated surface or fomites (Cai *et al.*, 2020). As of April 2022, there are over 511 million confirmed human cases and over 6.2 million human deaths and continuous outbreaks worldwide (WHO, 2022a). To date, there is no specific effective antiviral drugs to treat the SARS-CoV-2 infection or COVID-19 patients (Ashour *et al.*, 2022). The Vaccinations are considered as an important strategy for control and prevention of COVID-19 outbreak. In the present, various vaccine platforms have been developed to prevent SARS-CoV-2 infection, transmission and disease severity including inactivated viruses, live attenuated viruses, recombinant viral vector vaccines, DNA vaccines, mRNA vaccines and protein subunit vaccines (Khamees *et al.*, 2022).

1.4.3 Variant classification of SARS-CoV-2

As an RNA virus, SARS-CoV-2 has a high mutation rate. The RNA polymerase with the limitation of proof-reading function and genomic recombination while different variants infection in the same host, can generate the mutants or variant of viruses (Mandary *et al.*, 2019; Haddad *et al.*, 2021).

Currently, the diversity of SARS-CoV-2 genomes have been classified as lineage or clade by the nomenclature algorithm systems such as the Global Initiative on Sharing All Influenza Data (GISAID), the Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) and Nexstrain (Shu and McCauley, 2017; Hadfield *et al.*, 2018; Rambaut *et al.*, 2021). In general, most of nucleotide mutation or amino acid substitution result to little or no impact on the virus properties. However, some

mutation patterns can affect the viral phenotype regarding to viral transmissibility, disease severity and vaccine performance. These mutations have raised the public health concern and the viruses are labeled by the World Health Organization as the variants of concern (VOCs), the variants of interest (VOIs) and the variant under monitoring (VUM) (WHO, 2022c).

The variant of concern (VOC) of SARS-CoV-2 are designated based on 1) the mutations affecting increase transmissibility, 2) increase pathogenicity, and 3) decrease the efficiency of diagnostics, vaccines, and treatments. Up to date, WHO have assigned five VOCs including Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2) and Omicron (B.1.1.529) (WHO, 2022c).

Alpha variant (B.1.1.7)

The Alpha variant (B.1.1.7), was first reported in the United Kingdom in November 2020 (Kirby, 2021). The variant has amino acid substitutions on spike protein including N501Y, A570D, P681H, T716I, S982A and D1118H (O'Toole, 2022). The Alpha variant (B.1.1.7) has 43–82% more transmissibility than the Wuhan strain. The Alpha variant had spread and became dominant variant in May 2021 (Davies *et al.*, 2021; O'Toole, 2022).

Beta variant (B.1.351)

In October 2020, the Beta variant (B.1.351) with multiple mutations on spike gene including D80A, D215G, K417N, A701V, N501Y and E484K, were commonly reported in South Africa (O'Toole, 2022). This variant has more transmissible than the previous circulated virus (Abu-Raddad *et al.*, 2022). By January 2021, the Beta variant of SARS-CoV-2 was spread to other continents such as America, Europe, Asia and Australia (Tang *et al.*, 2021).

Gamma variant (P.1)

The gamma variant (P.1) emerged in Brazil in November 2020 with mutations containing on spike protein including L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, H655Y, T1027I (Faria *et al.*, 2021; O'Toole, 2022). Gamma variant has high transmissibility and increase immune evasion (Faria *et al.*, 2021).

Delta variant (B.1.617.2)

The Delta variant was first reported in India in December 2020 (Yang and Shaman, 2022). This variant contains the amino acid substitutions of spike protein including T19R, L452R, T478K, P681R, D950N (O'Toole, 2022). The Delta variant has increased viral replication, increased disease transmissibility due to high affinity binding between viral and host receptors, and decreased vaccine efficiency due to promote immune evasion (Bian *et al.*, 2021).

Omicron variant (B.1.1.529)

Omicron variant was identified in Botswana and South Africa in early November 2021 (WHO, 2021b). In the end of December 2021, Omicron variant outbreaks were reported in 154 countries worldwide (GISAID, 2022). This variant harbor high numbers of spike gene mutations including A67V T95I G339D S371L S373P K417N N440K G446S S477N T478K E484A Q493R G496S Q498R N501Y T547K D614G H655Y N679K P681H N764K D796Y N856K Q954H N969K (O'Toole, 2022). Omicron variant has 3.2 times higher transmissibility than Delta variant (Long *et al.*, 2022). However, Omicron variant is more likely to propagate at the upper respiratory tract than lower respiratory tract thus it causes less severity than previous variants (Lamers *et al.*, 2022).

1.4.4 SARS-CoV-2 in animals

The origins of SARS-CoV-2 remain unclear, but some research suggest that SARS-CoV-2 could have a zoonotic origin. The existing coronaviruses with the most

closely related to SARS-CoV-2 are bat and pangolin coronaviruses. The BatCoV RaTG13 from a horseshoe bat isolated in China in 2013 contains 96.2% whole genome similarity to SARS-CoV-2 (Zhou *et al.*, 2020). The pangolin coronavirus isolated from China in 2019 has a whole genome similarity of 91.02 percent to SARS-CoV-2, but the pangolin coronavirus's receptor binding domain (RBD) amino acid has a higher affinity for SARS-CoV-2 than the BatCoV (Zhang *et al.*, 2020b).

Since SARS-CoV-2 pandemic outbreak in human, several SARS-CoV-2 infections in animals have been reported due to spillover event from humans to animals. The first report of SARS-CoV-2 infection in animals was discovered in dogs in Hong Kong in February 2020 (Sit *et al.*, 2020). As of April 2022, SARS-CoV-2 in animals have been reported from 35 countries worldwide including the Americas, Europe, Asia and Africa with 23 animal species including cat, dog, mink, otter, ferret, lions, tiger, puma, snow leopard, gorilla, white-tailed deer, fishing cat, Binturong, South American coati, spotted hyena, Eurasian lynx, Canada lynx, hippopotamus, hamster, mule deer, giant anteater, West Indian manatee, black-tailed marmoset (OIE, 2022). Although most SARS-CoV-2 infections in animals are spread from people to animals, there have been reports of SARS-CoV-2 spillover from animals to humans in farmed minks and pet hamsters (Hammer *et al.*, 2021; Oude Munnink *et al.*, 2021; Yen *et al.*, 2022). SARS-CoV-2 transmission among animals were found in experimental studies including hamsters, ferrets, cats, raccoon dogs, deer mice and white-tailed deer (Freuling *et al.*, 2020; Gaudreault *et al.*, 2020; Schlottau *et al.*, 2020; Sia *et al.*, 2020; Fagre *et al.*, 2021; Martins *et al.*, 2022a). Animal to animal transmission may have occurred naturally in farmed minks and white-tailed deer (Chaintoutis *et al.*, 2021b; Hale *et al.*, 2022).

1.4.5 SARS-CoV-2 in Thailand

Thailand was the first country with a SARS-CoV-2 patient outside China on 13 January 2020. The first SARS-CoV-2 patient in Thailand was a Chinese visitor from

Wuhan China (WHO, 2020b). As of May 2022, there were over 4.3 million SARS-CoV-2 confirmed human cases with over 29,000 human deaths (WHO, 2022d). Up to date, at least five waves of SARS-CoV-2 outbreaks have been reported in Thailand since March 2020. The first wave occurred during March to June 2020 with total 3,042 cases and 57 deaths from lineage A.6 (Puenpa *et al.*, 2020; Joonlasak *et al.*, 2021). The second wave of outbreak from December 2020 to March 2021 emerged from the immigrant workers in Samut Sakhon province. There were approximately 22,000 SARS-CoV-2 infection human cases from this outbreak caused by dominant variant lineage B.1.36.16 or GH clade (Lerdsamran *et al.*, 2022). The Thailand's 3rd wave SARS-CoV-2 outbreak during April to June 2021, there were 58,613 reported cases with 422 deaths from Alpha variant (B.1.1.7) (WHO, 2022d). The fourth wave of COVID-19 outbreak, Delta variant (B.1.617.2) was started in May 2021. During 6 months of the this outbreak, there were 292,086 COVID-19 reported cases and 2,951 deaths from July to December 2021 (WHO, 2022d). The fifth wave of COVID-19 outbreak, VOC Omicron variant infection was reported since mid of December 2021. As of May 2022, there were 306,238 SARS-CoV-2 confirmed cases and 1,123 deaths, and the Omicron variant SARS-CoV-2 is still spreading in Thailand (WHO, 2022d).

Serological study of SARS-CoV-2 infection in animals in Thailand have been reported. In 2020, there was a study showed that 1.66% of dogs and cats in Bangkok and vicinities had seropositivity for nucleocapsid IgG of SARS-CoV-2 by ELISA test (Udom *et al.*, 2022). Furthermore, a bats coronavirus in Thailand has 95.86% genetic similarity to human SARS-CoV-2 and pangolins at wildlife checkpoint stations in Thailand in 2020 had neutralizing antibodies to SARS-CoV and SARS-CoV-2 (Wacharapluesadee *et al.*, 2021).

This dissertation presents our findings of the cross-sectional and retrospective SARS-CoV-2 surveillances in domestic animals and wildlife species in Thailand from 2017 to 2023 into separated five chapters including;

1) **Chapter 2:** SARS-CoV-2 surveillance in domestic animals during the second wave of the COVID-19 outbreak in Thailand which has been published in the topic **"Survey of SARS-CoV-2 in dogs and cats in high-risk areas during the second wave of COVID-19 outbreak, Thailand" in Zoonoses and Public Health, September 2022, Volume 69 Issue 6, pp 737-745**

2) **Chapter 3:** SARS-CoV-2 surveillance in domestic animals during the third wave of the COVID-19 outbreak in Thailand which has been published in the topic "**First cases of SARS-CoV-2 infection in dogs and cats in Thailand" in Transboundary and Emerging Diseases, July 2022 Volume 69 Issue 4, e979-e991**

3) **Chapter 4:** SARS-CoV-2 surveillance in domestic animals during the fourth wave of the COVID-19 outbreak in Thailand which has been published in the topic **"SARS-CoV-2 Delta variant infection in domestic dogs and cats, Thailand" in Scientific reports, May 2022 Volume 12 Issue 1, 8403**

4) **Chapter 5:** SARS-CoV-2 surveillance in wildlife in Thailand which is in the manuscript preparation in the topic **"SARS-CoV-2 surveillance in wildlife in Thailand and the infection in captive lions and a tiger"**

5) **Chapter 6:** Conclusions and Recommendations

CHAPTER 2

SARS-CoV-2 surveillance in domestic animals during the second wave of the COVID-19 outbreak in Thailand

This work has been published in the topic of

Survey of SARS-CoV-2 in dogs and cats in high-risk areas during the second wave of COVID-19 outbreak, Thailand

Zoonoses and Public Health, September 2022, Volume 69 Issue 6, pp 737-745

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2.1 Abstract

A cross-sectional survey of SARS-CoV-2 in domestic dogs and cats was conducted in high-risk areas, five subdistricts of Samut Sakhon Province, the epicenter of the second wave of the COVID-19 outbreak in Thailand in February 2021. A total of 523 swab samples (nasal, oral, and rectal swabs) and 159 serum samples from dogs (n = 83) and cats (n = 93) were collected and tested for SARS-CoV-2 RNA and antibodies. All swab samples tested negative for SARS-CoV-2 RNA by real-time RT-PCR with three panels of specific primers and probes. Although all dogs and cats were negative for SARS-CoV-2 RNA, 3.14% (5/159) had anti-N-IgG antibodies against SARS-CoV-2 by indirect multi-species ELISA. Our results demonstrated SARS-CoV-2 exposure in domestic animals living in high-risk areas during the second wave of the COVID-19 outbreak in Thailand. Thus, the use of one health approach for monitoring SARS-CoV-2 in domestic animals in high-risk areas of COVID-19 outbreaks should be routinely conducted and will provide benefits to risk communications in communities.

2.2 Introduction

Coronavirus disease of 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in late December 2019. COVID-19 is a pandemic disease in which over 213 million confirmed human cases with more than 4.4 million deaths have been reported worldwide (as of 26 August 2021) (WHO, 2022a). The first cluster of COVID-19 was associated with the seafood and wildlife market in China, and the whole genome sequences of the SARS-CoV-2 virus were closely related to coronaviruses from bats and pangolins (Li *et al.*, 2020; Zhou *et al.*, 2020). In Thailand, the first wave of the COVID-19 outbreak occurred from March to May 2020, with fewer than 3,100 confirmed human cases. The second wave of the COVID-19 outbreak from December 2020 to February 2021 reported more than 21,527 confirmed human cases, and Samut Sakhon Province was the epicenter of the second wave of the outbreak (WHO, 2021c). Evidence of SARS-CoV-2 spillover from infected humans to animals has been reported in dogs, cats, gorillas, leopard, lions, minks, otters, pumas and tigers (Abdel-Moneim and Abdelwhab, 2020; Newman *et al.*, 2020; Sailleau *et al.*, 2020; Sit *et al.*, 2020; OIE, 2021b; Ruiz-Arrondo *et al.*, 2021). The detection of SARS-CoV-2 RNA has been reported in dogs and cats in several countries such as Belgium, China, France, Spain, the UK, and the USA (Abdel-Moneim and Abdelwhab, 2020; Newman *et al.*, 2020; Sailleau *et al.*, 2020; Sit *et al.*, 2020; Ruiz-Arrondo *et al.*, 2021). Specific antibodies against SARS-CoV-2 in dogs and cats in close contact with COVID-19 patients and in affected areas have also been reported (Michelitsch *et al.*, 2020; Patterson *et al.*, 2020; Zhang *et al.*, 2020a). The transmission of SARS-CoV 2 from dogs/cats to humans has never been reported. However, SARS-CoV-2 transmission from animals (minks) to humans has been reported in the Netherlands (Oude Munnink *et al.*, 2021). Thus, the monitoring of SARS-CoV-2 in domestic animals in high-risk areas, especially those with close contact with owners, is important for risk communication in communities. In this study, we conducted a survey of SARS-CoV-2 in domestic

dogs and cats in high-risk areas, including field hospitals and COVID-19 positive households, in five subdistricts of Samut Sakhon Province, which were the epicenters of the second wave of the COVID-19 outbreak in February 2021. The samples from dogs and cats living in high-risk areas were tested for SARS-CoV-2 RNA and antibodies.

2.3 Materials and Methods

2.3.1 Study site

In this study, we conducted a cross-sectional survey of SARS-CoV-2 in domestic dogs and cats in February 2021. A high-risk area, Samut Sakhon Province, was selected because the province had the highest numbers of confirmed human cases during the second wave of the COVID-19 outbreak in Thailand. As of February 2021, more than 20,000 SARS-CoV-2 confirmed human cases (accounting for 95% of new cases in Thailand) were detected in the province reported by Thailand's Centre for COVID-19 Situation Administration (CCSA) (WHO, 2021c). Five subdistricts (Mahachai, Tha Chalom, Tha Sai, Krok Krak and Na Di) where the epicenters of the second wave of the COVID-19 outbreak in Thailand were included in the study (Figure 1). The second wave of the COVID-19 outbreak in Thailand was first reported in Samut Sakhon Province on 20 December 2020, by active case finding with 576 new cases related to the central shrimp market and was linked to immigrant workers at the market. On 7 February 2021, local and state authorities had announced Na Di and Tha Sai subdistricts as the controlled areas with high control measures, where approximately 7,000 confirmed human cases (mostly immigrant workers) from local factories tested positive for SARS-CoV-2. A lockdown was implemented in these two subdistricts to prevent high-risk persons from traveling. Restrictions were eased when the situation in these subdistricts had improved. At the epicenters, at least eight field hospitals were established for COVID-19 patients and close contact persons, including field hospitals in Mahachai, Krok Krak, Pantai Norasingh-1, Pantai Norasingh-2, Tha Chalom, Na Di-1, Na Di-2, and Tha Sai (CHI, 2021). We selected four out of eight field hospitals for sample collection from dogs and cats living near the field

hospitals. The high-risk areas for sample collection in this study included four field hospitals and COVID-19-positive households in five subdistricts of Samut Sakhon Province.

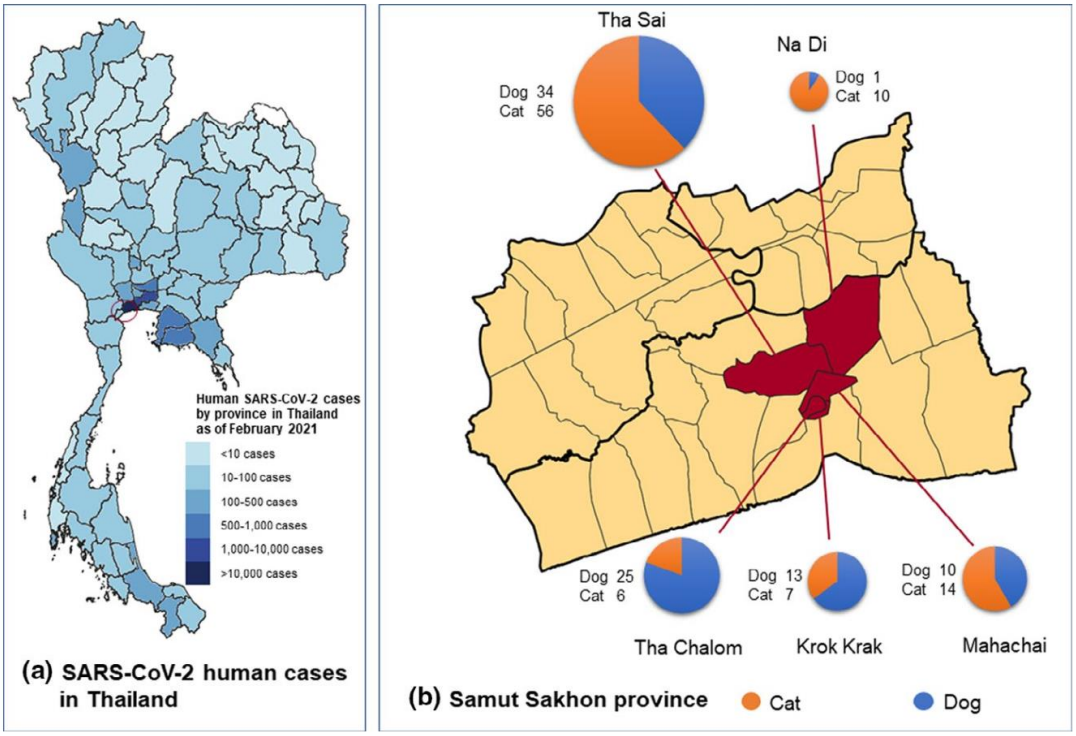

Figure 2.1 Study areas of SARS-CoV-2 survey in dogs and cats in high-risk areas during the second wave of COVID-19 outbreak of Thailand.

(a) A high-risk area, Samut Sakhon Province, reported the highest accumulated SARS-CoV-2 human cases in the second wave of the COVID-19 outbreak in Thailand (as of 24 Feb 2021). (b) Five subdistricts, Krok Krak, Mahachai, Na-Di, Tha-Chalom, and Tha-Sai, were selected for the SARS-CoV-2 survey in dogs and cats. Circles represent the location and number of dogs and cats sampled in this study.

2.3.2 Sample collection

Sample collection from dogs and cats was conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand (approval No. 2031035). The samplings were conducted according to the convenience or willingness of the owners or caretakers for COVID-19 testing. A cross-sectional sample collection from dogs and cats in high-risk areas, five subdistricts of Samut Sakhon Province, Thailand, was carried out on 9–16 February 2021. In total, we included 176 animals, including dogs (*n* = 83) and cats (*n* = 93). In detail, by subdistrict, the 176 animals were from Mahachai (*n* = 24), Tha Chalom (*n* = 31), Tha Sai (*n* = 90), Krok Krak (*n* = 20), and Na Di (*n* = 11). By type of sample, nasal swabs (*n* = 171), oral swabs (*n* = 176), rectal swabs (*n* = 176), as well as serum samples (*n* = 159) were collected from 176 animals (Table 2.1). Of animals in high-risk areas, 10 cats with a known history of close contact with COVID-19patients were identified. Nasal, oral, and rectal swabs were collected by nylon flocked swabs (Copan). Each swab was placed in the mixture of lysis buffer. Blood samples (2 ml) were collected from the cephalic or saphenous vein. The samples were transported at 4°C to the laboratory of the Center of Excellence for Emerging and Reemerging Infectious Diseases in Animals (CUEIDAs), Chulalongkorn University, within 24 hr. The samples were collected from dogs and cats of all ages with and without showing clinical signs. Some animals showed mild clinical signs (dogs; *n* = 6 and cats; *n* = 3), including nasal discharge (*n* = 8), occular discharge (*n* = 1), coughing (*n* = 6), sneezing (*n* = 2), vomiting (*n* = 2), and diarrhea (*n* = 2). The age of dogs ranged from 3 months to 20 years (average age 39.6 months), and the age of cats ranged from 3 months to 20 years (average age 36.9 months)

Table 2.1 Cross-sectional sample collection for SARS-CoV-2 in dogs and cats in 5 subdistricts of Samut Sakhon Province by location (field hospital and high-risk area) and type of sample (nasal swab, oral swab, rectal swab, and serum).

Location	Dogs				Cats			
	Nasal swab	Oral swab	Rectal swab	Serum	Nasal swab	Oral swab	Rectal swab	Serum
High risk area (Field hospitals and COVID-19 households)								
Mahachai	10	10	10	10	12	14	14	12
Tha Chalom	25	25	25	24	6	6	6	15
Tha Sai	34	34	34	31	55	56	56	40
Krok krak	13	13	13	13	6	7	7	5
Na Di	1	1	1	1	9	10	10	8
Total	**83**	**83**	**83**	**79**	**88**	**93**	**93**	**80**

2.3.3 Real-time RT-PCR for the detection of SARS-CoV-2 RNA

In this study, 523 swab samples of dogs and cats (176 animals) were subjected to RNA extraction. Viral RNA was extracted from individual nasal swabs (*n* = 171), oral swabs (*n* = 176), and rectal swabs (*n* = 176) using the IndiSpin® Pathogen Kit (Indical Bioscience GmbH) according to the manufacturer's instructions. For the detection of SARS-CoV-2 RNA, three panels of real-time RT-PCR assays were used. Panel A, real-time RT-PCR based on primers and probes specific to the E and RdRp genes following WHO recommendations was used (Corman *et al.*, 2020a). Panel B, real-time RT-PCR based on primers and probes specific to the N1 and N2 genes following CDC recommendations was used (CDC, 2020). Panel C, the commercial Angentex® COVID-19 qPCR detection kit specific to the N1, ORF1ab, and RdRp genes (After Lab Company) was used (Table 2.2). A one-step real-time RT-PCR assay was performed by a Superscript III One-Step RT-PCR System with Platinum Taq Polymerase (Invitrogen™). Samples with a Ct value of <36 were considered positive, while samples with a Ct value of 36–40 were considered suspected, and >40 were considered negative. In this study, the OIE definition was used for a confirmed animal case of SARS-CoV-2 infection, in which at least two specific targets tested positive (OIE, 2020). Positive RNA of canine respiratory coronavirus (CRCoV), canine enteric coronavirus (CECoV), feline coronavirus (FCoV), and cohort pre-COVID-19 RNA from dogs and cats were used to test the specificity of real-time RT-PCR assays. PCR specific to the GAPDH gene was also applied as an internal control for RNA extraction (Suwannakarn *et al.*, 2008).

Table 2.2 List of primers and probes used for SARS-CoV-2 detection in this study.

Target	Description	Oligonucleotide Sequence (5'>3')
Panel A	(Corman et al., 2020)	
RdRP gene	SARs-CoV-2 RdRp forward primer	5'- AGT GAR ATG GTC ATG TGT GGC G-3'
	SARs CoV RdRp reverse primer	5'-ARA TGT TAA ASA CAC TAT TAG CAT AA-3'
	SARs-CoV-2 RdRp probe	56-FAM/CCA GGT GGA ACC TCA TCA GGA GAT G/38 HQ_1
E gene	SARs-CoV-2 RdRp reverse primer	5'-CAG GTA CGT TAA TAG TTA ATA GCG TA-3'
	SARs CoV RdRp forward primer	5'-AAT ATT CGA GTA CGC ACA C-3'
	SARs-CoV-2 RdRp probe	56-FAM/CAC TAG CCA TCC TTA CTG CGC TTC GA/38 HQ_1
Panel B	(CDC, 2020)	
N1	2019-nCoV_N1 forward primer	GAC CCC AAA ATC AGC GAA AT
	2019-nCoV_N1 reverse primer	TCT GGT TAC TGC CAG TTG AAT CTG
	2019-nCoV_N1 probe	FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ
N2	2019-nCoV_N2 forward primer	TTA CAA ACA TTG GCC GCA AA
	2019-nCoV_N2 reverse primer	GCG CGA CAT TCC GAA GAA
	2019-nCoV_N2 probe	FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkFQ
Panel C	(Angentex®)	
N1	N1_F	(Angentex®)
	N1_R	(Angentex®)
	N1_probe	(Angentex®)
ORF1ab	ORF1ab_F	(Angentex®)
	ORF1ab_R	(Angentex®)
	ORF1ab_probe	(Angentex®)
RdRp	RdRp_F	(Angentex®)
	RdRp_F	(Angentex®)
	RdRp_probe	(Angentex®)

2.3.4. Indirect ELISA test for the detection of SARS-CoV-2 antibodies

An indirect enzyme-linked immunosorbent assay (ELISA), ID Screen® SARS-CoV-2 Double Antigen Multispecies ELISA kit (ID VET), was used for the detection of SARS-CoV-2 antibody. The ELISA detects the IgG antibodies against the nucleocapsid (N) protein of the SARS-CoV-2 virus in animal sera and was performed according to the manufacturer's recommendation. The optical density (OD) at 450 nm was read. The OD value of each sample was calculated as the S/P percentage (S/P%). The sample with S/P% ≥ 60% was considered positive, while the sample with S/P% between 50%–60% was considered suspected, and the sample with S/P% <50 was negative.

2.3.5 Surrogate virus neutralization test for the detection of SARS-CoV-2 antibodies

The cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech) was used and performed according to themanufacturer's recommendation. Absorbance reading at 450 nm was acquired using a microplate reader immediately following stop solution addition. The percentage inhibition (% inhibition) was calculated. The sample with % inhibition ≥20% indicated the presence of SARS-CoV-2 neutralizing antibodies, and % inhibition <20% was considered negative (Meyer *et al.*, 2020). The surrogate virus neutralization test (sVNT) assay detects SARS-CoV-2 antibodies according to the measurement of antibody-mediated inhibition of SARS-CoV-2 RBD-ACE2 interaction

2.3.6 Data analysis

Descriptive statistics were used to describe demographic information, locations, and types of samples from dogs and cats in this study. Fisher's exact test was used to analyse the difference in SARS-CoV-2 seropositivity and demographic information (sex, age, and location) of animals by the Epi Info™ 7 program (CDC)

Table 2.3 ELISA and sVNT tests for SARS-CoV-2 antibodies in pre-COVID-19 dog and cat sera.

No.	Host	Date	ELISA[a] (%S/P)	sVNT [b] (% Inhibition)
1	Dog	2014	1.43[c]	-
2	Dog	2014	3.26	-
3	Dog	2014	2.35	-
4	Dog	2014	3.49	-
5	Dog	2014	0.63	-
6	Dog	2014	3.95	-
7	Dog	2014	3.57	-
8	Dog	2014	13.35	-
9	Dog	2014	15.19	5.33
10	Dog	2014	1.22	-
11	Dog	2014	1.83	-
12	Dog	2014	0.61	-
13	Cat	2015	<0	-
14	Cat	2015	0.06	-
15	Cat	2015	0.63	-
16	Cat	2015	<0	-
17	Cat	2015	6.69	15.13
18	Cat	2015	2.69	-
19	Cat	2015	0.51	-
20	Cat	2015	0.41	-
21	Cat	2015	0.61	-
22	Cat	2015	2.04	-
4	Cat	2015	1.33	-
24	Cat	2015	1.22	-
25	Dog	2016	2.40	-
26	Dog	2016	-0.13	-
27	Dog	2016	-0.31	-
28	Dog	2016	1.35	-
29	Dog	2016	-0.04	-
30	Dog	2016	4.85	-
31	Dog	2017	0.57	-
32	Dog	2017	-0.22	-
33	Dog	2017	4.15	-
34	Dog	2017	0.22	-
35	Dog	2017	0.04	-
36	Dog	2017	11.39	-

No.	Host	Date	ELISA[a] (%S/P)	sVNT [b] (% Inhibition)
37	Dog	2019	22.57	-
38	Dog	2019	10.34	-
39	Dog	2019	0.92	-
40	Dog	2019	3.71	-
41	Dog	2019	0.31	-
42	Dog	2019	28.76	-
43	Cat	2017	-0.22	-
44	Cat	2017	0.13	-
45	Cat	2017	0.13	-
46	Cat	2017	-0.31	-
47	Cat	2017	0.22	-
48	Cat	2017	-0.22	-
49	Cat	2017	-0.13	-
50	Cat	2017	-0.13	-
51	Cat	2018	9.65	-
52	Cat	2018	-0.57	-
53	Cat	2018	0.04	-
54	Cat	2018	-0.57	-
55	Cat	2018	-0.31	-
56	Cat	2018	-0.31	-
57	Cat	2019	-0.31	-
58	Cat	2019	-0.31	-
59	Cat	2019	2.84	-
60	Cat	2019	-0.04	-
61	Cat	2019	0.65	-
62	Cat	2019	0.31	-
Range			-0.57-28.76	5.33-15.13
Median			0.61	-
Mean			2.72	12.89

[a] ELISA: The ID Screen® SARS-CoV-2 Double Antigen Multispecies ELISA kit (ID VET, Montpellier, France). The cut-off value, if S/P% > 60% was postive, S/P% 50 – 60 % was suspected, < 50% was negative.

[b]VNT (Virus neutralization): The cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, Jiangsu, China). The cut-off value, if % inhibition ≥ 20% was positive, <20% was negative.

[C] Udom et al., 2021

2.4 Results

In this study, SARS-CoV-2 RNA could not be detected in all nasal, oral, and rectal swab samples of dogs and cats. At least three panels of primers and probes for real-time RT-PCR were used for the detection of SARS-CoV 2 RNA, and all real-time RT-PCR assays (Panels A, B, and C) showed the same result (Table 2.4). The serum samples (*n* = 159) of dogs and cats were subjected to indirect multispecies ELISA. Our results showed that 3.14% (5/159) of serum samples had SARS-CoV-2 antibodies (Table 2.5). The ELISA test showed positive and suspected results with %S/P 51.89 to 119.16 (Figure 2.2). The ELISA-positive samples were also subjected to sVNT using the cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit. Among the five serum samples examined, none showed positive neutralization antibodies with % inhibition of 1.763%–18.527% (mean 11.84%) (Table 2.5). It should be noted that positive (*n* = 4) and suspected (*n* = 1) serum for SARS-CoV-2 antibodies was collected from dogs living in high-risk areas around the field hospitals in three subdistricts. The serum was from healthy dogs that were mixed breeds and between 1.5 and 7 years of age (Table 2.6). In this study, known pre-COVID-19 serum samples from dogs (*n* = 30) and cats (*n* = 32) collected from 2014–2019 were also included in the ELISA test, and all pre-COVID-19 serum samples showed negative results for SARS-CoV-2 antibodies (Table 2.3). There was no statistical significance among seropositive dogs regarding the sex, age, and location of the animals.

Table 2.4 Detection of SARS-CoV-2 RNA in dogs and cats by real-time RT-PCR specific to E, RdRp (Panel A); N1, N2 (Panel B); and N1, ORF1ab, RdRp (Panel C)

	Dogs			Cats		
Location	**E and RdRp[a]**	**N1and N2[b]**	**N1, ORF1ab, RdRp[c]**	**E and RdRp[a]**	**N1and N2[b]**	**N1, ORF1ab, RdRp[c]**
High-risk area (Field hospital and COVID-19 household)						
Mahachai	0/30	0/30	0/30	0/40	0/40	0/40
Tha Chalom	0/75	0/75	0/75	0/18	0/18	0/18
Tha sai	0/102	0/102	0/102	0/167	0/167	0/167
Krok krak	0/39	0/39	0/39	0/20	0/20	0/20
Na Di	0/3	0/3	0/3	0/29	0/29	0/29
Total	0/249	0/249	0/249	0/274	0/274	0/274

[a]Set of primers and probes specific to the E and RdRp genes of SARS-CoV-2 following WHO recommendations (Corman *et al.*, 2020a).

[b]Set of primers and probes specific to the N1 and N2 genes of SARS-CoV-2 following the CDC recommendations (CDC, 2020).

[c]Set of primers and probes specific to the N1, ORF1ab, and RdRp genes of SARS-CoV-2 by Angentex® COVID-19 qPCR detection kit (After Lab Company)

Table 2.5 Seroprevalence of SARS-CoV-2 in dogs and cats in Samut Sakhon Province by indirect ELISA and sVNT.

	ELISA[a]		sVNT[b]	
Location	Dogs	Cats	Dogs	Cats
High-risk area (Field hospitals and COVID-19 households)				
Mahachai	1[c]/10	0/12	0/1	-
Tha Chalom	3/34	0/15	0/3	-
Tha Sai	0/21	0/40	-	-
Krok Krak	1/13	0/5	0/1	-
Na Di	0/1	0/8	-	-
Total	5[c]/79	0/80	0/5	0/0

[a]ELISA: ID Screen® SARS-CoV-2 Double Antigen Multispecies ELISA kit (ID VET). The cutoff value was as follows: if S/P% ≥ 60% was positive, S/P% 50%–60% was suspected, and <50% was negative.

[b]sVNT: Surrogate virus neutralization test.

[c]The sample with suspected ELISA result (S/P% was 51.89).

Table 2.6 Description of animals with positive antibodies for SARS-CoV-2 by indirect ELISA test and sVNT test.

								Real-time Rt-PCR			ELISA[a]	sVNT[b]
Animal	District	Location	Species	Breed	Sex	Age	Clinical signs	E and RdRp	N1 and N2	N1, ORF1ab, RdRp	(S/P%)	(%inhibition)
D20	Mahachai	Field Hospital	Dog	Mixed	Male	5 years	Healthy	Neg[c]	Neg	Neg	51.89	1.763
D30	Krok Krak	Field Hospital	Dog	Mixed	Male	7 years	Healthy	Neg	Neg	Neg	101.43	10.693
D54	Tha Chalom	Field Hospital	Dog	Mixed	Female	>2 years	Healthy	Neg	Neg	Neg	85.53	18.527
D61	Tha Chalom	Field Hospital	Dog	Mixed	Female	>4 years	Healthy	Neg	Neg	Neg	119.16	17.822
D70	Tha Chalom	Field Hospital	Dog	Mixed	Female	1.5 years	Healthy	Neg	Neg	Neg	65.50	6.22

[a]ELISA: ID Screen® SARS-CoV-2 Double Antigen Multispecies ELISA kit (ID VET). The cutoff value was as follow: if S/P% ≥60% was positive, S/P% 50%–60% was suspected, and <50% was negative.

[b]VNT (virus neutralization): The cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech). The cutoff value, if % inhibition ≥20% was positive, <20% was negative.

[c]Neg; Negative result or BDL, below the detection limit.

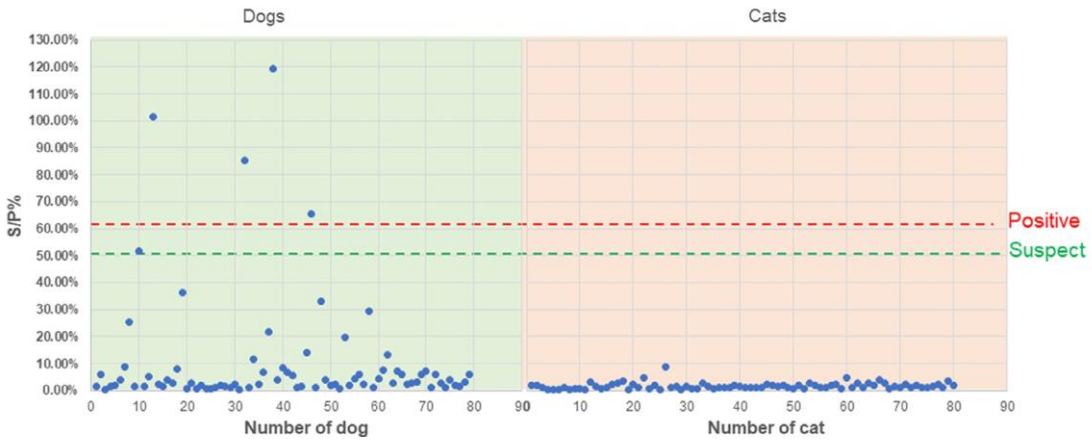

Figure 2.2 Positive and suspected sera with antibodies against the N protein of SARS-CoV-2 from dogs and cats living in high-risk areas by indirect multispecies ELISA.

It is interesting to note that one rectal swab from a 3-month-old cat living in a high-risk area (Na Di subdistrict) showed a suspected result (high Ct > 36) of real-time RT-PCR specific to the N1 and N2 genes of SARS-CoV-2 (Panel B) but negative real-time RT-PCR specific to the RdRp and E genes (Panel A) and commercial SARS-CoV-2 test kit (Panel C). The serum sample of the cat collected on February 2021 tested negative by ELISA (1.73% S/P) and sVNT assay (<20% % inhibition). To further confirm the suspected sample, the RNA sample was subjected to ARTIC primer set amplifications and Nanopore® sequencing. PCR amplification and Nanopore® sequencing failed to amplify and sequence any SARS-CoV-2 genome. We revisited and collected samples from a cat in April 2021, and all samples tested negative by both real-time RT-PCR and ELISA tests. Thus, the result of SARS-CoV-2 detection in a cat is inconclusive. Ten cats with a known history of close contact with COVID-19 positive patients were identified, carefully restrained, and sampled. All cats showed no clinical signs and tested negative for SARS-CoV-2 RNA by real-time RT-PCR (three panel sets) in the laboratory. Similar to SARS-CoV-2 RNA detection, all cats were negative for SARS-CoV-2 antibodies by both indirect ELISA and sVNT (Table 2.7).

Table 2.7 Detection of SARS-CoV-2 RNA and antibodies in animals in close contact with COVID-19 positive households.

Household	District	Species	Breed	Sex	Age	Clinical signs	Real-time RT-PCR			ELISA	sVNT
							E and RdRp[a]	N1 and N2[b]	N1, ORF1ab, RdRp[c]		
A	Tha Chalom	Cat	DSH	Intact female	>5 year	Healthy	0/1	0/1	0/1	0/1	0/1
		Cat	DSH	Intact female	>1 year	Healthy	0/1	0/1	0/1	0/1	0/1
		Cat	DSH	Intact female	>1 year	Healthy	0/1	0/1	0/1	0/1	0/1
		Cat	DSH	Intact female	>1 year	Healthy	0/1	0/1	0/1	0/1	0/1
B	Mahachai	Cat	DSH	Neutered female	3 year	Healthy	0/1	0/1	0/1	0/1	0/1
		Cat	DSH	Neutered female	>5 year	Healthy	0/1	0/1	0/1	0/1	0/1
		Cat	DSH	Neutered female	>2 year	Healthy	0/1	0/1	0/1	0/1	0/1
		Cat	DSH	Neutered female	>2 year	Healthy	0/1	0/1	0/1	0/1	0/1
C	Tha Sai	Cat	DSH	Neutered female	3 year	Healthy	0/1	0/1	0/1	0/1	0/1
D	Tha Sai	Cat	DSH	Intact female	6 months	Healthy	0/10	0/10	0/10	0/10	0/10

[a]Primers and probes specific to the E and RdRp genes of SARS-CoV-2 following the WHO recommendations (Corman *et al.*, 2020a).

[b]Primers and probes specific to the N1 and N2 genes of SARS-CoV-2 following the Centers for Disease Control and Prevention recommendations (CDC, 2020).

[c]Primers and probes specific to the N1, ORF1ab, and RdRp genes of SARS-CoV-2 following the commercial Angentex® COVID-19 qPCR detection kit.

2.5 Discussion

This study is the first to report a virological survey of SARS-CoV-2 in domestic dogs and cats in Thailand. A serological survey of SARS-CoV-2 in domestic dogs and cats during hospital visits in Thailand was recently reported by our research group (Udom *et al.*, 2022). Samut Sakhon Province was considered as an epicenter of the second wave of COVID-19 outbreaks in Thailand. The province is composed of three districts (A; Mueang, B; Krathum Baen and C; Ban Phaeo), of which district A (Mueang district) is composed of 18 subdistricts. Of 18 subdistricts, five subdistricts with more than 95% of COVID-19 patients, which are the epicenters of the second wave of COVID-19 outbreaks, were selected for the SARS-CoV-2 survey in domestic animals. At least eight field hospitals have been established for COVID-19 patients in Samut Sakhon Province. We selected five subdistricts that are the hot spots of COVID-19 outbreaks where most human cases were reported at the markets, factories, and communities with high numbers of immigrant workers. Two subdistricts, Na Di and Tha Sai, were announced as controlled areas with high control and strict measures (WHO, 2021c). In this study, SARS-CoV-2 RNA could not be detected in any dogs or cats in high-risk areas both around field hospitals and in COVID-19 households. At least three panels of primers and probes were used to detect SARS-CoV-2 RNA, and the results from all three panels were the same. Since SARS-CoV-2 RNA could not be detected in this study, the possible explanation is that natural infection with SARS-CoV-2 in animals is less likely or the time of virus shedding and cross-sectional sample collection vary among animals (Newman *et al.*, 2020; Patterson *et al.*, 2020). For example, a study showed that natural SARS-CoV-2-infected dogs and cats can shed the viruses in nasal swabs for 13–16 days (Newman *et al.*, 2020; Sit *et al.*, 2020). In the experimental setting, dogs and cats can shed the virus only at 2–6 days post-inoculation (dpi) (from rectal swabs) and 6 dpi (from nasal washes) (Shi *et al.*, 2020). In contrast to SARS-CoV-2 RNA detection in swab samples, serological results showed that 3.14% of domestic animals living in high-risk areas

had antibodies against SARS-CoV-2, suggesting that the animals might have been exposed to unknown sources of SARS-CoV-2. Our findings agree with previous reports of SARS-CoV-2 surveys in dogs and cats. A serological survey in Italy reported that 3.3% of dogs and 5.8% of cats had SARS-CoV-2 antibodies by a using plaque reduction neutralization test (Patterson *et al.*, 2020). In Wuhan, China, a serological survey revealed 14.7% SARS-CoV-2 seropositivity in cats in high-risk areas using ELISA based on RBD (Xiong *et al.*, 2020). In Germany, only 0.69% of cats had SARS-CoV-2 antibodies by RBD detection ELISA (Michelitsch *et al.*, 2020). In Thailand, our recent report on serological surveys during hospital visits showed that only 1.21% of dogs and cats had anti-N-IgG antibodies against SARS-CoV-2 (Udom *et al.*, 2022). This demonstrates that dogs and cats in high-risk areas such as Samut Sakhon, the epicenters of the second wave of COVID-19 outbreaks, have high risks of SARS-CoV-2 exposure. Up to date, information on the prevalence of SARS-CoV-2 antibodies in animals based on N-based ELISA was limit. A study in northern Italy reported N-based ELISA for the detection of SARS-CoV-2 antibodies in cats with high sensitivity and specificity which 1 out of 105 cat samples tested positive and none of 136 pre-pandemic serum samples tested positive for SARS-CoV-2 antibodies (Spada *et al.*, 2021). Another study conducted in the USA showed that there was relatively good correlation between the N-based ELISA and the sVNT (Barua *et al.*, 2021). A study in France showed N-based ELISA for SARS-CoV-2 antibodies in cats correlated well with S-based ELISA and neutralization test (PRNT) excepting in early stage of infection (7 days after clinical symptoms) (Natale *et al.*, 2021). A recent study on the sensitivity and specificity of both S- and N-based commercially available assays showed at least 98% agreement (Ainsworth *et al.*, 2020). Despite good correlation among tests, antigenic cross-reactivity, and diagnostic errors of the N- and S-based ELISA on animal samples should not be ignored. A limitation of our study is the discrepancy between the results of N protein-based ELISA and sVNT. Similar observations have been documented in serological survey of SARS-CoV-2 antibodies in dogs and cats (Barua

et al., 2021; Spada *et al.*, 2021). It has also been reported that the spike protein-based ELISA better correlates with the neutralization assay than the N protein-based ELISA (Folegatti *et al.*, 2020; Ni *et al.*, 2020; Okba *et al.*, 2020). Therefore, it was not unexpected that the positive and suspected serum (*n* = 5) based on N protein-based ELISA in this study lacked neutralizing activity. To date, the role of domestic dogs and cats in the maintenance and transmission of SARS-CoV-2 from pets to humans is uncertain. There is no evidence of viral transmission from pet animals to humans (Decaro *et al.*, 2021a). However, reports of SARS-CoV-2 transmission from minks to humans have been documented (Oude Munnink *et al.*, 2021). Thus, public education regarding the risks of close contact of pet owners and their pets should be provided. Owners from COVID-19-positive households should monitor and test their pets for SARS-CoV-2. Recommendations on the health monitoring of pets in the households could be useful during COVID-19 outbreaks. For example, a study reported early detection of SARS-CoV-2-infected cats with mild respiratory signs, while the individuals in the household did not show any clinical signs of COVID-19 (Newman *et al.*, 2020). In summary, this study provides evidence of SARS-CoV-2 antibodies in domestic dogs and cats living in high-risk areas of COVID-19 in Thailand. Our study supports the use of the one health approach on emerging infectious diseases such as COVID-19 to improve human and animal health. The results from the SARS-CoV-2 survey in domestic animals at the epicenter or high-risk areas of the COVID-19 outbreak provide benefits to risk communications in the communities. For example, animal health consultations, information sheets, and COVID-19 in animal hotlines regarding the knowledge and practices of COVID-19 in animals have been successfully implemented to advocate people in communities, especially in the epicenter of the COVID-19 outbreak and could be used as a model for future COVID-19 outbreaks.

CHAPTER 3

SARS-CoV-2 surveillance in domestic animals during the third wave of the COVID-19 outbreak in Thailand

This work has been published in the topic of

First cases of SARS-CoV-2 infection in dogs and cats in Thailand

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3.1 Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the coronavirus disease 2019 (COVID-19) pandemic in humans since late 2019. Here, we investigated SARS-CoV-2 infection in dogs and cats during COVID-19 quarantine at private veterinary hospitals in Thailand. From April to May 2021, we detected SARS-CoV-2 in three out of 35 dogs and one out of nine cats from four out of 17 households with confirmed COVID-19 patients. SARS-CoV-2 RNA was detected from one of the nasal, oral, rectal and environmental swabs of dog-A (15 years old, mixed breed, male dog), cat-B (1 year old, domestic shorthair, male cat), dog-C (2 years old, mixed breed, female dog) and dog-D (4 years old, Pomeranian, female dog). The animals tested positive for SARS-CoV-2 RNA from 4 to 30 days after pet owners were confirmed to be COVID- 19 positive. The animals consecutively tested positive for SARS-CoV-2 RNA for 4 to 10 days. One dog (dog-A) showed mild clinical signs, while the other dogs and a cat remained asymptomatic during quarantine at the hospitals. SARS-CoV-2 specific neutralizing antibodies were detected in both the dogs and cat by surrogate virus neutralization tests. Phylogenetic and genomic mutation analyses

of whole genome sequences of three SARS-CoV-2 strains from the dogs and cat revealed SARS-CoV-2 of the Alpha variant (B.1.1.7 lineage). Our findings are suggestive of human-to-animal transmission of SARS-CoV-2 in COVID-19-positive households and contamination of viral RNA in the environment. Public awareness of SARS-CoV-2 infection in pet dogs and cats in close contact with COVID-19 patients should be raised.

Keywords: cat, dog, infection, SARS-CoV-2, Thailand

3.2 Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is a pandemic disease. As of 16 June 2021, more than 175 million confirmed human cases have been reported, with over 3.81 million deaths (WHO, 2022a). Evidence of SARS-CoV-2 spillover from humans to animals has been reported in dogs, cats, tigers, lions, gorillas and minks (McAloose *et al.*, 2020; Newman *et al.*, 2020; Sit *et al.*, 2020). SARS-CoV-2 infection in domestic dogs and cats has been reported in 22 countries in America, Europe and Asia (Decaro *et al.*, 2021a; OIE, 2021b). Cats are susceptible to SARS-CoV-2 infection and can show mild-to-moderate respiratory symptoms, while dogs developed no or mild respiratory symptoms (McAloose *et al.*, 2020; Sailleau *et al.*, 2020; Segalés *et al.*, 2020). In Thailand, the current outbreak (3rd wave) of COVID-19 started in late March 2021, and the number of confirmed human cases of SARS-CoV-2 infection is still rising (WHO, 2021a). During the outbreak, pet dogs and cats of COVID-19-positive patients were optionally quarantined at university and private veterinary hospitals. In this study, we collected swab samples (nasal, oral and rectal swabs) from 35 dogs and nine cats from COVID-19-positive households and examined them for SARS CoV-2 infection in those animals. We identified SARS-CoV- 2 infection in three dogs and one

cat by virological testing, serological testing and viral genome analysis. This study is the first to report dogs and cats infected with SARS-CoV-2 in Thailand.

3.3 Materials and Methods

3.3.1 Sample collection from dogs and cats

In this study, we investigated SARS-CoV-2 infection in domestic dogs and cats quarantined at private animal hospitals during the third wave of the COVID-19 outbreak reported by Thailand's Centre for COVID- 19 Situation Administration (CCSA) (WHO, 2021a). From March to May 2021, we collected samples from dogs (*n* = 35) and cats (*n* = 9) from 17 households located in Bangkok and the vicinity (Table 3.1). It is noted that all animals lived indoor. The sample collection was conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand (approval No. 2031035). The sampling of dogs and cats was conducted according to the convenience or willingness for COVID-19 testing of owners and animal hospital staff. In total, nasal swabs (*n* = 58), oral swabs (*n* = 61) and rectal swabs (*n* = 93) from 35 dogs and nine cats were collected. Serum samples (*n* = 9) were collected from SARS-CoV-2-positive animals. Nasal, oral and rectal swabs were collected by using flocked nylon swabs (Copan, California, USA). Environmental samples including hair/body swabs (*n* = 11), water container swabs (*n* = 11) and floor swabs (*n* = 11) were collected from cages of COVID-19 positive pets at the animal hospitals. Environmental sampling was conducted before animal sampling and daily cleaning with disinfectant. Each swab was placed in 1 ml of RNAprotect® Tissue Reagent (Qiagen, Hilden, Germany). Blood samples (1–2 ml) were collected from the cephalic or saphenous vein. The samples were transported to the laboratory of the Center of Excellence for Emerging and Re-emerging Infectious Diseases in Animals (CUEIDAs), Chulalongkorn University, within 24 h.

Table 3.1 List of COVID-19 positive households from which dogs and cats were sampled in this study.

Household	Date	District	Province	# of COVID-19 patients in household	SARS-CoV-2 detection (Positive sample/total sample)			SARS-CoV-2 detection (Positive animal/total animal)	
					Nasal swab	Oral swab	Rectal swab	Dog	Cat
1	Mar 2021	Lak si	Bangkok	NA	0/1	0/1	0/1	-	0/1
2	Apr 2021	Prawet	Bangkok	2	0/2	0/2	0/9*	0/2	-
3	Apr 2021	Rachathewi	Bangkok	1	0/2	0/2	0/2	-	0/1
4	Apr 2021	Lat Krabang	Bangkok	NA	0/2	0/2	0/12*	0/1	0/1
5 (A)[a]	Apr 2021	Bang Khae	Bangkok	2	8/8 [a]	7/8 [a]	1/8 [a]	1/1 [a]	-
6	Apr 2021	Chatuchak	Bangkok	1	-	-	0/3*	0/1	-
7	May 2021	Bang Khae	Bangkok	NA	0/2	0/2	0/2	0/2	-
8 (B)[b]	May 2021	Mueang	Samut Parkan	3	2/4 [b]	3/4 [b]	1/4 [b]	-	1/1[b]
9	May 2021	Phra Khanong	Bangkok	1	0/20	0/20	0/20	0/20	-
10	May 2021	Mueang	Nonthaburi	NA	0/1	0/1	0/1	0/1	-
11	May 2021	Prawet	Bangkok	NA	0/2	0/2	0/2	0/1	0/1
12 (C)[c]	May 2021	Bangkok Noi	Bangkok	3	2/4 [c]	1/4 [c]	0/4 [c]	1/1 [c]	-
13	May 2021	Don Mueang	Bangkok	NA	0/3	0/3	0/3*	0/1	-
14 (D)[d]	May 2021	Bang Khen	Bangkok	5	1/6 [d]	1/6 [d]	0/6 [d]	1/2 [d]	-
15	May 2021	Nong Khae	Sara Buri	NA	-	-	0/12*	-	0/4
16	May 2021	Mueang	Samut Parkan	2	-	0/3	0/3*	0/1	-
17	May 2021	Mueang	Samut Sakhon	1	0/1	0/1	0/1	0/1	-
			Total		**13/58**	**12/61**	**2/93**	**3/35**	**1/9**

NA; Not available but at least 1 COVID-19- patient in the household

*Sample collection on more than 1 visit

[a] Dog-A (CU27042) from household A, 6 visits for sample collection

[b] Cat-B (CU27081) from household B, 4 visits for sample collection

[c] Dog-C (CU27184) from household C, 3 visits for sample collection

[d] Dog-D (CU27186) from household D (high Ct Value), 3 visits for sample collection

3.3.2 Detection of SARS-CoV-2 RNA by real-time RT-PCR

The swab samples, including nasal swabs (*n* = 58), oral swabs (*n* = 61) and rectal swabs (*n* = 93), were subjected to RNA extraction by using the magnetic bead-based automatic purification equipment of a GENTi™ 32 - Automated Nucleic Acid Extraction System (GeneAll®, Seoul, South Korea). In brief, the swab sample was vigorously vortexed for at least 15 s before removing the swab. Next 200 μl of supernatant was mixed with 7 μl of RNA carrier and then added to an extraction tube. The RNA extraction process was performed according to the manufacturer's instructions. Finally, 50 μl of viral RNA was obtained from the RNA extraction process.

For the detection of SARS-CoV-2, real-time RT-PCR based on primers and probes specific to the *E* and *RdRp* genes following WHO recommendations was used (Corman *et al.*, 2020a), and primers and probes specific to the *N1* and *N2* genes following the Centers for Disease Control and Prevention recommendations were also used (CDC, 2020) (Table 3.2). A one-step real-time RT-PCR assay was performed by using a Superscript III One-Step RT-PCR System with Platinum Taq Polymerase (Invitrogen™, California, USA). In brief, a total 25 μl reaction contained 2 μl of RNA, 12.5 μl of 2X reaction buffer of the SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen™, California, USA), 1 μl of reverse transcriptase/Platinum Taq, 0.8 mM $MgSO_4$, 0.8 μM each primer and probe and RNase-free water. Thermal cycling was performed at 50°C for 15 min for reverse transcription, followed by 95°C for 2 min and then 45 cycles of 95°C for 15 s, and 60°C for 30 s for the *N1* and *N2* genes. For the *E* and *RdRP* genes, thermal cycling was performed at 55°C for 10 min for reverse transcription, followed by 95°C for 3 min and then 45 cycles of 95°C for 15 s and 58°C for 30 s. Samples with a *Ct* value of <36 were considered positive, while samples with a *Ct* value of 36–40 were considered suspected and those with a *Ct* value > 40 were considered negative (CDC, 2020). In this study, we used the World Organisation for Animal Health (WOAH or OIE) definition for a confirmed case of

animal SARS-CoV-2 infection, in which at least two specific targets (genomic regions) tested positive, indicating SARS-CoV-2 positivity (OIE, 2020).

Table 3.2 List of the primers and probes used for SARS-CoV-2 detection in this study.

Target	Description	Oligonucleotide Sequence (5'>3')	References
RdRp gene	SARS-CoV-2 RdRp forward primer	5'- GTG ARA TGG TCA TGT GTG GCG G-3'	(Corman *et al.*, 2020b)
	SARS CoV-2 RdRp reverse primer	5'- CAR ATG TTA AAS ACA CTA TTA GCA TA-3'	
	SARS-CoV-2 RdRp probe	56-FAM/CAG GTG GAA CCT CAT CAG GAG ATG C/38 HQ_1	
E gene	SARS-CoV-2 E forward primer	5'-ACA GGT ACG TTA ATA GTT AAT AGC GTA-3'	(Corman *et al.*, 2020b)
	SARS CoV-2 E reverse primer	5'-ATA TTG CAG CAG TAC GCA CAC A-3'	
	SARS-CoV-2 E probe	56-FAM/ACA CTA GCC ATC CTT ACT GCG CTT CG/38 HQ_1	
N1	2019-nCoV_N1 forward primer	GAC CCC AAA ATC AGC GAA AT	(CDC, 2020)
	2019-nCoV_N1 reverse primer	TCT GGT TAC TGC CAG TTG AAT CTG	
	2019-nCoV_N1 probe	FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ	
N2	2019-nCoV_N2 forward primer	TTA CAA ACA TTG GCC GCA AA	(CDC, 2020)
	2019-nCoV_N2 reverse primer	GCG CGA CAT TCC GAA GAA	
	2019-nCoV_N2 probe	FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkFQ	

3.3.3 Detection of SARS-CoV-2 antibodies by indirect enzyme-linked immunosorbent assay (indirect ELISA) and virus neutralization test (VNT)

We used an ID Screen® SARS-CoV-2 Double Antigen Multispecies ELISA Kit (ID VET, Montpellier, France) to detect SARS-CoV-2 antibodies in serum samples. This indirect ELISA was based on the detection of anti-SARS-CoV-2 nucleocapsid antibodies (IgG) in the tested animal serum and was performed according to the manufacturer's instructions (Sailleau *et al.*, 2020). Briefly, 25 μl of each serum sample and positive and negative control samples were transferred to separate wells, diluted with 25 μl of dilution buffer, incubated at 37°C for 45 min and washed five times with 300 μl of washing buffer. After washing, 100 μl of horseradish peroxidase (HRP)-conjugated N protein recombinant antigen was added and incubated at 25°C for 30 min. Then, the wells were washed five times with 300 μl of washing buffer. After washing, 100 μl of the substrate was added to each well and incubated at 25°C for 20 min. Then, 100 μl of stop solution was added to stop the reaction. The optical density (OD) at 450 nm of each sample was read. The OD of each sample was calculated as the S/P percentage (S/P%). Serum with S/P% > 60% was defined as positive, while serum with S/P% 50%-60% was considered suspected.

To detect the presence of SARS-CoV-2-neutralizing antibodies, sera of dogs and cats were subjected to sVNTs by using a cPass™ SARS-CoV- 2 Neutralization Antibody Detection Kit (GenScript Biotech, Jiangsu, China). The assay detects neutralizing antibodies for the interaction between the virus receptor-binding domain (RBD) and the ACE2 cell surface receptor (Tan *et al.*, 2020). Briefly, 50 μl of each 1:10-diluted serum sample was mixed with 50 μl of horseradish peroxidase conjugated to the SARS-CoV-2 spike RBD (HRP-RBD) and incubated at 37°C for 30 min. After dilution, each mixture was added to each well precoated with ACE2 protein and incubated at 37°C for 15 min. Then, the wells were washed 4 times with 260 μl of washing buffer. After washing, TMB solution was added and incubated at 25°C for 15 min. Then, 50 μl of stop solution was added. The OD at 450 nm of each

well was read. The OD of each sample was calculated as the inhibition percentage (% inhibition); serum with % inhibition above 20% was considered positive, and serum with % inhibition not exceeding 20% was considered negative (Meyer *et al.*, 2020).

3.3.4 Characterization and phylogenetic analysis of SARS-CoV-2

We performed whole-genome sequencing of 3 SARS-CoV-2 strains by Oxford Nanopore sequencing. All gene segments of SARS-CoV-2 were amplified by ARTICS nCoV-2019 sequencing protocol V3 (LoCost). Briefly, 8 μl of undiluted RNA was mixed with 2 μl of LunaScript® RT SuperMix (NEB, Ipswich, MA, USA) and incubated at 25°C for 2 min, 55 °C for 10 min and 95 °C for 1 min for cDNA synthesis. The SARS-CoV-2 primer scheme was used to perform two pools of multiplex PCRs by usingQ5® Hot StartHigh-Fidelity DNA polymerase (NEB, MA, USA) according to the ARTIC protocol. For the ARTIC multiplex PCR, thermal cycling was set at 98°C for 30s and then 35 cycles of 98°C for 15s and 65°C for 5 min. After ARTIC multiplex PCR, library preparation was performed following the Oxford Nanopore rapid sequencing kit (SQK-RAD004) manufacturer's instructions and Midnight SARS-CoV-2 genome sequencing protocol. In brief, PCR products of pools 1 and 2 were mixed (10 μl of pool 1 and 10 μl of pool 2) and 7.5 μl of the mixture was used for binding with 2.5 μl of fragmentation mix from an Oxford Nanopore rapid sequencing kit. After incubation at 30°C for 1 min, 80°C for 1 min and 4°C for 30 s, the product was cleaned up by AMPure XP Bead Cleanup (Beckman Coulter, CA, USA) in a 1:1 ratio and eluted with 10 mM Tris-HCl pH 8.0. One microliter of rapid adapter was added, and the mixture was loaded into a flow cell (Oxford Nanopore MinION device) and run under MinKNOW (v19.12.5) software (Baker *et al.*, 2021). The output reads from the Oxford Nanopore MinION device were filtered using the sequencing summary file under the following parameters: minimum read length ≥ 500 nt and read quality ≥7. The reads that passed the parameters were converted from "Fast5" into "Fastq" format using

the GPU version of the Nanopore Guppy basecaller (v3.4.4) tool. Genome assembly was conducted by using the genome detective program (Vilsker *et al.*, 2019). De-novo approach with CLC Genomics Workbench Bio assembly software v11.0.1 (CLC Bio, 2005, Denmark) was used for nucleotide data analysis. Whole-genome nucleotide sequences of SARS-CoV- 2 from the dogs and cat were then submitted to the GenBank database under the accession numbers MZ396818, MZ401455 and MZ414173.

Phylogenetic analysis of SARS-CoV-2 was performed by comparing with nucleotide sequences of 942 genomes (at least 29,000 base pairs in length) isolated from Thailand from January 2020 to May 2021. The genome sequences were selected and downloaded from the GISAID database. The 5′ and 3′ untranslated regions were trimmed with at least 95% reference genome coverage and retained (Wuhan-Hu-1). The dataset was aligned using the MAFFT FFT-NS-2 algorithm and default parameter settings (Katoh *et al.*, 2002). A neighbour-joining tree was constructed by using MEGA program v7.0 (Tempe, AZ, USA) with the maximum composite likelihood substitution model and bootstrapping with 1,000 replicates. Lineage classification was performed by using the Pangolin tool (Rambaut *et al.*, 2021). Genome mutation analysis of SARS-CoV-2 was performed based on variant classifications and definitions (CDC) (Kumar *et al.*, 2016; CDC, 2021). Genome positions were based on the reference genome sequence of Wuhan-Hu-1 (MN908947).

3.3.5 Data analysis

Descriptive statistics were used to describe demographic information, locations and types of samples from dogs and cats in this study. Frequencies and percentages were used to report SARS-CoV-2 infection in animals and households. Phylogenetic analysis was performed by the neighbor-joining algorithm with the maximum composite likelihood substitution model and bootstrapping with 1,000 replicates by the MEGA program.

3.4 Results

All nasal, oral and rectal swab samples from dogs and cats (*n* = 44) from 17 households of COVID-19-positive patients were screened for SARS-CoV-2 RNA by real-time RT-PCR with specific primers and probes for the *N1, N2, E* and *RdRp* genes. A total of three out of 35 dogs (8.6%) and one out of nine cats (11.1%) from four out of 17 (23.53%) households were positive for SARS-CoV-2 RNA confirmed by real-time RT-PCR. The dogs and cat were followed up for nasal, oral, rectal and environmental (hair, water containers, floor) sample collection at quarantine animal hospitals until all tests were negative (Table 3.3, Table 3.5, and Figure 3.1).

Dog-A (CU27042) is a 15-year-old, 20 kg, mixed breed, castrated male dog. The animal had pre-existing clinical disorders, including degenerative mitral value disease (stage B1), multiple hepatic masses and fibrosarcoma at the right metatarsus. The owners of dog-A were a 50-year-old man and 45-year-old woman who were diagnosed with COVID-19 on 23 April 2021 (owner A1) and 26 April 2021 (owner A2). On 29 April 2021, dog-A visited Chulalongkorn University Small Animal Hospital for surgery for right hind limb amputation. Nasal, oral and rectal swabs were collected, and the animal was transferred to a private animal hospital for post-operative care. Additional sample collection for SARS-CoV-2 detection was carried out on six further occasions at the private animal hospital. A blood sample was collected on six occasions for serological testing. During quarantine, the dog developed mild clinical signs, including nasal discharge, sneezing and labored breathing (on 5–8 May 2021) (Table 3.4). We detected SARS-CoV-2 RNA from nasal and oral swabs of dog-A by real-time RT-PCR in six consecutive sample collections between 3 and 12 May 2021. A high viral load (low *Ct* value) was observed in nasal and oral swabs of the dog on 4–9 May 2021 (Ct 15.67–34.59) and in all environmental samples (hair, water container and floor) between 4 and 12 May 2021 (Ct 26.32–35.58) (Table 3.5).

Cat-B (CU27081) is a 1-year-old, 3 kg, domestic-shorthair intact male cat. The animal was healthy and transferred to a private animal hospital on 7 May 2021.

There were three members in household B. The owner of cat-B was a 35-year-old male (owner B1) who tested positive for SARS-CoV-2 on 8 April 2021. An additional two members, an 85-year-old male (owner B2) and 80-year-old female (owner B3), were diagnosed with COVID-19 on 30 April 2021. Owners B1 and B2 passed away after hospitalization in April and in May 2021, respectively. Swab samples from cat-B were collected on four occasions between 7 and 19 May 2021. During quarantine, the cat did not show any clinical signs. Nasal, oral and rectal swabs tested positive for SARS-CoV-2 RNA on the first sample collection (7 May 2021) (Ct 26.69–33.60). A nasal swab was tested positive on the next sample collection (9 May 2021) (Ct 32.37–34.73). High *Ct* values (low viral loads) were detected from water container swabs on 12 May 2021 (Ct 35.71) (Table 3.5).

Dog-C (CU27184) is a 2-year-old, 6 kg, mixed breed spayed female dog. The dog was transferred to a private animal hospital on 19 May 2021, after the owner (owner C1), a 26-year-old male, tested positive for COVID-19 on 15 May 2021. Other members of household C, two adult females (owners C2 and C3), were diagnosed with COVID-19 on 17 May 2021. Patient C1 was hospitalized and died in May 2021. Specimens were collected from dog-C on three occasions between 19 and 25 May 2021. Dog-C was healthy during the quarantine period. Nasal swabs tested positive at the first two visits (19 and 22 May 2021) (Ct 28.18–35.05). Environmental samples (hair and floor) showed high *Ct* values on 22 May 2021 (Ct 32.88–34.01) (Table 3.5).

Dog-D (CU27186) is a 4-year-old, 2.75 kg, Pomeranian, spayed female dog. The dog was transferred to a private animal hospital on 19 May 2021. There were five members in household D. The owner of the dog (owner D1), an 18-year-old female, tested positive for COVID-19 on 16 May 2021. All members of household D, adult males and females (owners D2-D5) were also diagnosed with COVID-19. Swab samples were collected from dog-D on three occasions between 19 and 25 May 2021. Nasal swabs tested positive with a high *Ct* value (32.46–34.42) at the first visit

(19 May 2021) and oral swab was positive (Ct 33.94) at the second visit (22 May 2021). Rectal and environmental samples tested negative (Table 3.5).

Serum samples were collected from the dogs and cat positive for SARS-CoV-2 were tested for anti-SARS-CoV-2 antibodies by indirect multispecies ELISA (indirect ELISA) and surrogate virus neutralization test (sVNT). Serum from dog-A was positive by indirect ELISA at 79.66%S/P and 70.97%S/P (20–21 days after owner-A1 tested positive, respectively). Dog-A developed neutralizing antibodies with 42.39%-60.55% inhibition (17 days after owner-A1 tested positive). Serum from CatB, collected on 12 May 2021, was positive by both indirect ELISA (87.20%) and sVNT (90.15%) (35 days after owner B1 tested positive). Dog-C had neutralizing antibodies with 37.20% inhibition (20 days after owner C1 tested positive). Dog-D had neutralizing antibodies with 32.17% inhibition (27 days after owner D1 tested positive). Control dog and cat sera, pre-COVID-19 sera, feline coronavirus (FCoV)-positive sera and sera from animals positive for canine respiratory coronavirus (CRCoV) and canine enteric coronavirus (CECoV) tested negative by both indirect ELISA and sVNT (Table 3.6).

In this study, we characterized three SARS-CoV-2 isolates from two dogs and a cat. Viral RNA from the nasal swabs of dog-A (Ct 15.67-22.19) on 4 May 2021, cat-B (Ct 26.69-29.75) on 7 May 2021 and dog-C (Ct 28.46-35.05) on 19 May 2021 was subjected to whole-genome sequencing by Nanopore sequencing using the ARTIC primer set. The RNA from dog-D (Ct 32.46-36.59) was unsuccessfully sequenced due to the low viral titre. The SARS-CoV-2 genome sequences were obtained from dog-A (29,778 nt), cat-B (29,713 nt) and dog-C (29,743 nt) and submitted to the GenBank database under the accession numbers MZ396818, MZ401455 and MZ414173, respectively (Table 3.7). We compared the whole genome sequences of SARS-CoV-2 from the dogs and cat with 942 of full-length sequences of the viruses from Thailand. The Thai SARS-CoV-2 clustered with the viruses of the Alpha variant (B.1.1.7 lineage) which is the predominant lineage of the recent third wave of COVID-19 outbreaks in Thailand (Figure 3.2). Analysis of genomic mutations of SARS-CoV-2

showed that the viruses from the dogs and cat contained mutations resembling the viruses of the B.1.1.7 lineage but differing from those of different lineages (B.1.36.16, A.6 and Wuhan-Hu-1) (Table 3.8).

Table 3.3 Description of the dogs and cat positive for SARS-CoV-2 and COVID-19-positive households.

ID	District	Location	Species	Breed	Sex	Age	Clinical signs	Date of first detection	COVID-19 positive household (# of owner)	SARS-CoV-2 sequence (bp)	GenBank accession #
Dog-A CU27042	Bang Khae	Bangkok	Dog	Mixed	Male	15 Years	Nasal discharge, Sneezing	3 May 21	Household-A (2) A1. 50-year-old male A2. 45-year-old female	WGS (29778 bp)	MZ396818
Cat-B CU27081	Mueang	Samut Prakarn	Cat	Domestic shorthair	Male	1 Year	Healthy	7 May 21	Household-B (3) B1. 35-year-old male B2. 85-year-old male B3. 80-year-old female (2 deaths: B1, B2)	WGS (29713 bp)	MZ401455
Dog-C CU27184	Bangkok Noi	Bangkok	Dog	Mixed	Female	2 Years	Healthy	19 May 21	Household-C (3) C1. 26-year-old male C2. > 60-year-old female C3. > 80-year-old female (1 death; C1)	WGS (29743 bp)	MZ414173
Dog-D CU27186	Bang Khen	Bangkok	Dog	Pomranian	Female	4 Years	Healthy	19 May 21	Household-D(5) D1. 18 -year-old female D2. >40-year-old female D3. >50-year-old male D.4> 70-year-old female D5. >70-year-old male	ND	ND

Table 3.4 Clinical signs of a SARS-CoV-2 infected dog (dog-A) in this study.

Animal	Clinical signs				
	Fever	Nasal discharge	Sneezing	Coughing	Dyspnea
Dog-A					
29 Apr 21	N/A	-	-	-	-
3 May 21	No (101.2°F)	-	-	-	-
4 May 21	No (100.2°F)	-	-	-	-
5 May 21	No (101.4°F)	+Yes	+Yes	-	-
6 May 21	No (101.2°F)	+Yes	+Yes	-	-
7 May 21	No (100.2°F)	+Yes	-	-	-
8 May 21	No (101.4°F)	+Yes	-	-	-
9 May 21	No (100.0°F)	-	-	-	-
10 May 21	No (100.2°F)	-	-	-	-
11 May 21	No (100.0°F)	-	-	-	-
12 May 21	No (100.0°F)	-	-	-	-

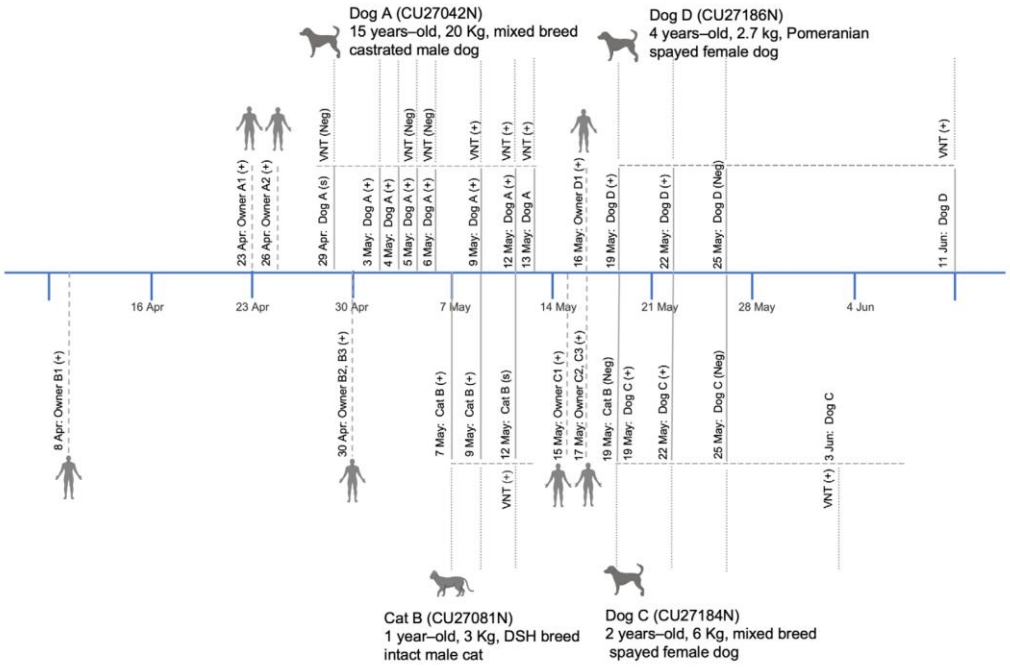

Figure 3.1 Timeline of SARS-CoV-2 detection in dogs and cats in the study.

Table 3.5 SARS-CoV-2 detection in the dogs and cat by real-time RT-PCR specific to N1, N2, E and RdRp.

Dog A												
	Rea-ltime RT-PCR (Ct value)											
	Nasal swab				Oral swab				Rectal swab			
Date	N1[a]	N2[a]	E[b]	RdRp[b]	N1	N2	E	RdRp	N1	N2	E	RdRp
29 Apr 21	-	-	-	-	s (37.30)	-	-	-	s (37.18)	-	-	-
3 May 21	+ (32.39)	S (38.14)	-	-	+ (27.88)	+ (33.11)	+ (32.02)	+ (33.05)	-	-	-	-
4 May 21	+ (15.67)*	+ (18.45)*	+ (18.87)*	+ (22.19)*	+ (25.56)	+ (31.04)	+ (28.77)	+ (31.41)	-	-	-	-
5 May 21	+ (15.59)	+ (16.77)	+ (19.10)	+ (21.67)	+ (28.39)	+ (33.30)	+ (33.20)	+ (33.44)	-	-	-	-
6 May 21	+ (26.61)	+ (30.68)	+ (29.01)	+ (28.04)	+ (29.73)	+ (34.54)	+ (31.59)	+ (31.25)	-	-	-	-
9 May 21	+ (30.66)	s (36.09)	+ (32.62)	+ (33.06)	+ (34.59)	-	+ (34.44)	-	-	-	-	-
12 May 21	+ (31.93)	s (38.82)	-	-	-	-	-	-	-	-	-	-
	Hair/body swab				Water container swab				Floor swab			
	N1	N2	E	RdRp	N1	N2	E	RdRp	N1	N2	E	RdRp
29 Apr 21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3 May 21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4 May 21	+(29.11)	s (38.20)	+(31.81)	+(34.37)	+(33.44)	-	+(33.97)	-	+(27.95)	+(33.38)	+(31.81)	+(34.01)
5 May 21	+(29.29)	s (38.33)	+(32.48)	+(33.87)	+(30.27)	+(34.72)	+(33.08)	+(34.86)	+(26.32)	+(32.45)	+(30.64)	+(32.57)
6 May 21	+(29.86)	s (37.35)	+(32.17)	-	s (36.13)	-	-	-	s (38.73)	-	-	-
9 May 21	+(34.04)	s (39.47)	+(34.43)	-	-	-	-	-	+(35.58)	-	+(35.13)	-
12 May 21	+(31.99)	-	-	-	-	-	-	-	s (37.19)	-	-	-
Cat-B												
	Real-time RT-PCR (Ct value)											
	Nasal swab				Oral swab				Rectal swab			
Date	N1[a]	N2[a]	E[b]	RdRp[b]	N1	N2	E	RdRp	N1	N2	E	RdRp
7 May 21	+(26.69)	+(31.06)	+(28.86)	+(29.75)	+(33.60)	s (38.95)	+(32.71)	-	+(30.90)	-	-	-
9 May 21	+(32.37)	-	+(34.0)	+(34.73)	s (37.80)	s (39.72)	-	-	-	-	-	-
12 May 21	s (36.39)	-	-	-	-	-	-	-	-	-	-	-
19 May 21	-	-	-	-	-	-	-	-	-	-	-	-
	Hair/body swab				Water container swab				Floor swab			
	N1	N2	E	RdRp	N1	N2	E	RdRp	N1	N2	E	RdRp
7 May 21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9 May 21	-	-	-	-	s (37.28)	-	-	-	-	-	s (36.73)	-
12 May 21	s (37.38)	-	-	-	+(35.71)	-	-	-	-	-	-	-
19 May 21	-	-	-	-	-	-	-	-	-	-	-	-
Dog-C												
	Real-time RT-PCR (Ct value)											
	Nasal swab				Oral swab				Rectal swab			
Date	N1[a]	N2[a]	E[b]	RdRp[b]	N1	N2	E	RdRp	N1	N2	E	RdRp
19 May 21	+(28.46)	s (38.03)	+(31.85)	+(35.05)	-	-	-	-	-	-	-	-
22 May 21	+(28.18)	s (39.21)	s (36.02)	s (36.38)	+(31.22)	-	-	-	-	-	-	-
25 May 21	-	-	-	-	-	-	-	-	-	-	-	-
	Hair/body swab				Water container swab				Floor swab			
	N1	N2	E	RdRp	N1	N2	E	RdRp	N1	N2	E	RdRp
19 May 21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
22 May 21	+(34.01)	-	-	-	-	-	-	-	+(32.08)	-	-	-
25 May 21	-	-	-	-	-	-	-	-	-	-	-	-

Dog-D												
	Real-time RT-PCR (Ct value)											
	Nasal swab				Oral swab				Rectal swab			
Date	N1[a]	N2[a]	E[b]	RdRp[b]	N1	N2	E	RdRp	N1	N2	E	RdRp
19 May 21	+(32.46)	-	+(34.42)	s (36.59)	-	-	-	-	-	-	-	-
22 May 21	-	-	-	-	+(33.94)	-	-	-	-	-	-	-
25 May 21	-	-	-	-	-	-	-	-	-	-	-	-
	Hair/body swab				Water container swab				Floor swab			
	N1	N2	E	RdRp	N1	N2	E	RdRp	N1	N2	E	RdRp
19 May 21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
22 May 21	-	-	-	-	-	-	-	-	-	-	-	-
25 May 21	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a]Primers and probes specific to the N1 gene and N2 gene of SARS-CoV-2 following the Centers for Disease Control and Prevention recommendations (CDC, 2020).

[b]Primers and probes specific to the E and RdRp genes of SARS-CoV-2 following the WHO recommendations (Corman *et al.*, 2020a).

NA; Not available.

Table 3.6 Anti-N IgG antibodies and neutralizing antibodies in the SARS-CoV-2-infected dogs and cat.

ID	Host	Date	ELISA [a]		sVNT [b]	
			OD	%SP	OD	% inhibition
Dog-A						
CU27042	Dog	29 Apr 21	0.115	5.10%	2.455	0.47%
CU27042	Dog	5 May 21	0.138	7.10%	2.506	-1.60%
CU27042	Dog	6 May 21	0.227	14.90%	2.411	2.25%
CU27042	Dog	9 May 21	0.694	55.70% (s)	1.421	42.39% (+)
CU27042	Dog	12 May 21	0.889	79.66% (+)	0.973	60.55% (+)
CU27042	Dog	13 May 21	0.798	70.96% (+)	1.017	58.77% (+)
Cat-B						
CU27081	Cat	12 May 21	1.055	87.20% (+)	0.243	90.15% (+)
Dog-C						
CU27184	Dog	3 June 21	0.43	35.82%	1.549	37.20% (+)
Dog-D						
CU27186	Dog	11 June 21	0.086	2.96%	1.673	32.17% (+)

Control serum

ID	Host	Date	ELISA [a]		sVNT [b]	
			OD	%SP	OD	% inhibition
Pre-COVID-19	Dog	2014	0.192	15.19%	2.513	5.33%
Pre-COVID-19	Dog	2016	0.072	1.40%	2.378	3.59%
Pre-COVID-19	Cat	2015	0.104	6.69%	2.352	4.64%
Pre-COVID-19	Cat	2017	0.058	0.13%	2.206	10.56%
FCoV	Cat	2020	0.059	0.22%	2.213	10.28%
FCoV	Cat	2020	0.074	1.50%	2.382	3.43%
CRCoV	Dog	2021	0.059	0.20%	2.381	3.47%
CRCoV	Dog	2021	0.081	2.10%	2.481	-0.59%
CECoV	Dog	2021	0.163	9.30%	2.363	4.20%
CECoV	Dog	2021	0.125	6.00%	2.401	2.66%

[a] ELISA: ID Screen® SARS-CoV-2 Double Antigen Multispecies ELISA Kit (ID VET, Montpellier, France). For the cutoff values, S/P% ≥60% was positive, S/P% 50–60 % was suspected, and S/P%< 50% was negative.

[b] VNT (virus neutralization): cPass^{TM} SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, Jiangsu, China). The cutoff values were defined as follows; positive if % inhibition ≥ 20% is positive and negative otherwise.

Table 3.7 Detail information of Oxford Nanopore MinION sequencer result of SARS-CoV-2 in this study.

Virus	GenBank accession No.	No. of reads	Average length (bp)	Total nucleotide (bp)	Genome size (bp)	Coverage (x)
CU 27042N	MZ396818	15,269	327.42	4,999,425	29,903	167.19
CU 27081N	MZ401455	39,705	319.57	12,688,661	29,903	424.33
CU 27184N	MZ414173	88,529	335.37	29,689,630	29,903	992.86

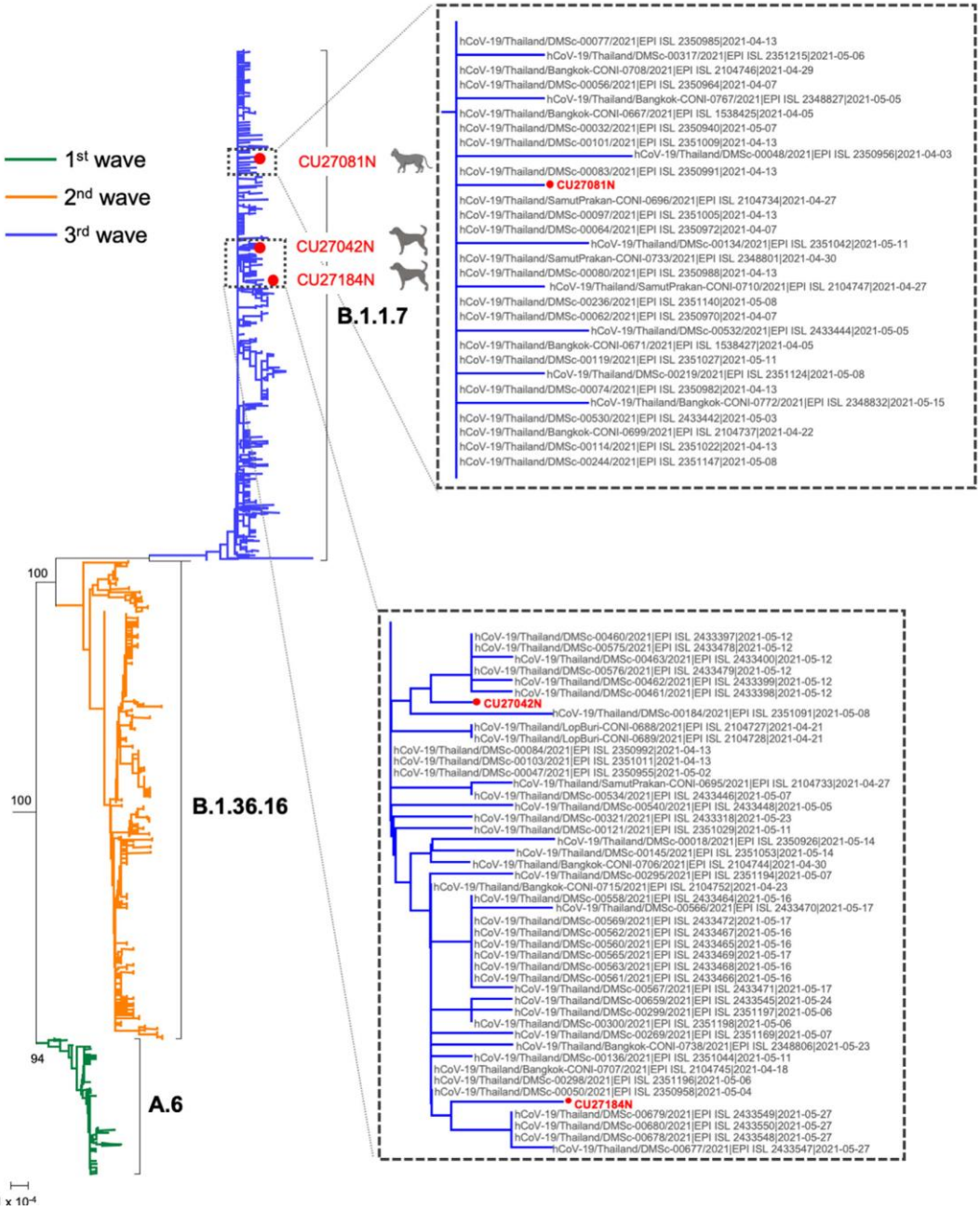

Figure 3.2 Phylogenetic analysis of SARS-CoV-2 obtaining from dogs and cats in Thailand during April to May 2021.

Whole genome sequences of SARS-CoV-2 from the dogs and cat Phylogenetic tree of 942 full-length SARS-CoV-2 isolates from Thailand retrieved from the GISAID database and three whole genome sequences of SARS-CoV-2 from the dogs and cat. The viruses isolated during the first, second and third waves are presented as green lines [first wave; n = 144], orange lines [second wave; n = 2550] and blue lines [third wave; n = 543]. The brackets indicate the lineages as A.6, B.1.36.16 and B.1.1.7. The tree was rooted by using the Wuhan-Hu-01 isolate. The scale bar indicates nucleotide substitutions per site.

Table 3.8 Genomic mutations of SARS-CoV-2 from the dogs and cat compared to those of SARS-CoV-2 in Thailand during the first, second and third waves.

CU27042N, CU27081N and CU27184N are the SARS-CoV-2 isolates characterized in this study. DMSc-00376/2021, DMSc-00181/2021 and DMSc-00478/2021 represent viruses from the third wave. CU-617/2021, CU-632/2021 and CU-646/2021 represent viruses from the second wave. Bangkok-CONI-0109/2020, Bangkok-CONI-0290/2020 and Bangkok-CONI-0304/2020 represent viruses in the first wave.

			ORF1a				ORF1b	Spike									ORF8				N		
Strain	Host	Lineage	1001	1708	2230	3675-3677	314	69-70	144	501	570	614	681	716	982	1118	27	52	68	73	3	203-204	235
Wuhan-Hu-1	Human	B	T	A	I	SGF	P	HV	S	N	A	D	P	T	S	D	Q	R	K	Y	D	RG	S
CU27042N	Dog	B.1.1.7	I	D	T	deletion	L	deletion	deletion	Y	D	G	H	I	A	H	stop	I	stop	C	L	KR	F
CU27081N	Cat	B.1.1.7	I	D	T	deletion	L	deletion	deletion	Y	D	G	H	I	A	H	stop	I	stop	C	L	KR	F
CU27184N	Dog	B.1.1.7	I	D	T	deletion	L	deletion	deletion	Y	D	G	H	I	A	H	stop	I	stop	C	L	KR	F
DMSc-00376/2021	Human	B.1.1.7	I	D	T	deletion	L	deletion	deletion	Y	D	G	H	I	A	H	stop	I	stop	C	D	KR	F
DMSc-00181/2021	Human	B.1.1.7	I	D	T	deletion	L	deletion	deletion	Y	D	G	H	I	A	H	stop	I	stop	C	L	KR	F
DMSc-00478/2021	Human	B.1.1.7	I	D	T	deletion	L	deletion	deletion	Y	D	G	H	I	A	H	stop	I	stop	C	L	KR	F
CU-617/2021	Human	B.1.36.16	T	A	I	SGF	L	HV	S	N	A	G	P	T	S	D	Q	R	K	Y	D	RG	S
CU-632/2021	Human	B.1.36.16	T	A	I	SGF	L	HV	S	N	A	G	P	T	S	D	Q	R	K	Y	D	RG	S
CU-646/2021	Human	B.1.36.16	T	A	I	SGF	L	HV	S	N	A	G	P	T	S	D	Q	R	K	Y	D	RG	S
Bangkok-CONI-0109/2020	Human	A.6	T	A	I	SGF	P	HV	S	N	A	D		T	S	D	Q	R	K	Y	D	RG	S
Bangkok-CONI-0290/2020	Human	A.6	T	A	I	SGF	P	HV	S	N	A	D	P	T	S	D	Q	R	K	Y	D	RG	S
Bangkok-CONI-0304/2020	Human	A.6	T	A	I	SGF	P	HV	S	N	A	D	P	T	S	D	Q	R	K	Y	D	RG	S

*Genome positions are based on the reference genome sequence of Wuhan-Hu-1 (MN908947).

3.5 Discussion

We reported SARS-CoV-2 infection in three dogs and one cat in Thailand. One animal (dog-A) showed illness with mild respiratory signs, but the other animals did not display any clinical symptoms and did not show any important blood chemistry abnormalities (Table 3.9). Similar to other studies, the infected dogs and cat showed non-specific and mild respiratory signs such as nasal discharge, sneezing, coughing and inappetence (Calvet *et al.*, 2021; Klaus *et al.*, 2021). During quarantine and follow-up visits, no animal died from viral infection, even though dog-A had pre-existing underlying diseases. It remains unclear whether infected dogs can transmit the virus to other animals, while cat to cat transmission has been observed in an experimental setting (Bosco-Lauth *et al.*, 2020; Shi *et al.*, 2020). It should be noted that our results showed a high viral load (low Ct value) in environmental samples from dog-A during quarantine at the hospital. Thus, contamination of SARS-CoV-2 in the environment and possible transmission from contaminated areas should not be ignored. Contamination of SARS-CoV-2 from animals to the environment, such as the fur and floor, has been reported in some studies (Oreshkova *et al.*, 2020; Klaus *et al.*, 2021). Stability of SARS-CoV-2 on the surface have been reported that viable virus could be detected up to 72 h on the surfaces (Van Doremalen *et al.*, 2020). Unfortunately, in this recent study, virus isolation was not performed due to the limitation of laboratory facility and the permission on live-virus propagation. Thus, shedding of infectious or viable virus from dogs and cats could not be confirmed.

This study is the first to report SARS-CoV-2 infection in domestic dogs and cats in Thailand. Our results suggested that dogs and cats can acquire viral infections from households with SARS-CoV-2-infected humans. Similarly, in previous reports, SARS-CoV-2 infection in dogs and cats in households with COVID-19 patients has been reported in many countries (Barrs *et al.*, 2020; Gaudreault *et al.*, 2020; Musso *et al.*, 2020; Newman *et al.*, 2020; Sailleau *et al.*, 2020; Segalés *et al.*, 2020; Sit *et al.*, 2020; Calvet *et al.*, 2021; Ruiz-Arrondo *et al.*, 2021). In this study, the frequency of

SARS-CoV-2 positivity in dogs (8.6%), cats (11.1%) and households (23.5%) was lower than that in previous studies in Brazil, China and the United States based on similar diagnostic assays (Barrs *et al.*, 2020; Hamer *et al.*, 2020; Calvet *et al.*, 2021), but higher than that reported in some countries (Sailleau *et al.*, 2020; Sit *et al.*, 2020; Ruiz-Arrondo *et al.*, 2021). There was a limitation that only rectal swabs could be collected from some households, which could have resulted in missing some infected animals in our investigation. In contrast, serial sample collection from animals provided more opportunity to detect SARS-CoV-2. For example, dog A showed suspected results in oral and rectal swabs at the first visit, but after six additional visits, the nasal, oral and environmental swabs tested positive for SARS-CoV-2. Our findings support the importance of longitudinal sample collection for the investigation of SARS-CoV- 2 infection in pet animals. Regarding the persistence of SARS-CoV-2 RNA, the dogs consecutively tested positive for SARS-CoV-2 RNA for 4 to 10 days (dog-A, 10 days; dog-C, 4 days and dog-D, 4 days), while cats consecutively tested positive for 6 days (cat-B, 6 days). In contrast, in previous studies, SARS-CoV-2 RNA has been observed for 14 to 31 days (in Brazil), 13 days (in China) and 25 days (in the United States) after the first positive sample (Hamer *et al.*, 2020; Shi *et al.*, 2020; Sit *et al.*, 2020; Calvet *et al.*, 2021). The dogs and cat in this study tested positive for SARS-CoV-2 RNA from 5 to 30 days after the index COVID-19 owner tested positive, which is comparable to that in studies in Brazil (11 to 51 days), China (28 days) and the United States (32 days) (Hamer *et al.*, 2020; Shi *et al.*, 2020; Sit *et al.*, 2020; Calvet *et al.*, 2021).

Infected dogs and cats develop antibodies against SARS-CoV-2 as early as 7 to 14 days post infection. The seropositivity of dogs in a previous study varied but was higher than that in cats (Barrs *et al.*, 2020; Hamer *et al.*, 2020; Patterson *et al.*, 2020). In this study, we used the parameter of the first pet owner positive by real-time PCR as day 1 (index case). Dog-A and cat-B developed anti-N IgG antibodies according to indirect ELISA, and 100% of animals developed neutralizing antibodies

according to sVNT. Dog-A and cat-B had neutralizing antibodies as early as 17 days and 35 days, respectively. For dog-C and dogD, serum samples were collected 20 to 27 days after the owner C1 and D1 positive for SARS-CoV-2, and we detected anti-SARS-CoV-2 antibodies in the serum as expected. Cat-B had the highest antibody level among the animals tested (87.20% by ELISA and 90.15% by sVNT), which reflects the timing for serum sample collection in which the animal might have been exposed to COVID-19 owners since 8 April 2021 (35 days after the index case, owner B1 positive). In addition, felines can develop high antibody titers, as demonstrated in previous studies (Barrs *et al.*, 2020; Hamer *et al.*, 2020; Patterson *et al.*, 2020). It should be noted that the discrepancy between the result of ELISA and sVNT had been observed. It has been reported that the N-protein based ELISA is less correlated with the neutralization assay (Folegatti *et al.*, 2020; Ni *et al.*, 2020; Okba *et al.*, 2020; Decaro *et al.*, 2021b; Decaro *et al.*, 2022). Therefore, it was not unexpected that the negative serum of dog-C (35.82%) and dog-D (2.96%) based on N protein-based ELISA had neutralizing activity (dog-C; 37.20% and dogD; 32.17%).

Phylogenetic analysis was performed on three whole genome sequences from the dogs (dog-A, dog-C) and cat (cat-B). Unfortunately, the limitation of this study is that the samples from SARS-CoV-2- positive pet owners were not available due to limits on access of human sample collection at state quarantine facilities. Instead, the phylogenetic analysis included the full-length sequences of SARS-CoV-2 from Thailand (n = 942), which are publicly available on the Global Initiative on Sharing All Influenza Data (GISAID) database. The whole genome sequences of dog-A, cat-B and dog-C were clustered with human SARS-CoV-2 of the Alpha variant (B.1.1.7 lineage). The phylogenetic tree clearly demonstrated that the B.1.1.7 lineage was a predominant lineage of the recent third wave of COVID-19 outbreaks in Thailand. According to the genome comparison of SARS-CoV-2, the SARS-CoV-2 isolates from the dogs and cat showed all mutations in agreement with human SARS-CoV-2 of the B.1.1.7 lineage (DMSc-00376/21, DMSc- 00181/21, DMSc-00478/21). All genomic

mutations (21 positions) in the ORF1a, ORF1b, spike, ORF8 and N genes were identical for canine, feline and human isolates of the B.1.1.7 viruses lineage analyzed in the study. The mutations in the spike gene conformed to the CDC classification and definitions of the B.1.1.7 lineage (alpha was proposed for the WHO label on 15 June 2021), which is a variant of concern (CDC, 2021; WHO, 2022c). In other studies, SARS-CoV-2 infection in pet dogs and cats from various SARS-CoV-2 lineages has been reported such as the B.1.1.39 lineage in cats in Switzerland (Klaus *et al.*, 2021), the B.1.177 lineage in a dog in Italy (Decaro *et al.*, 2021b) and clades G, GH and GR in dogs and cats in the United States (Hamer *et al.*, 2020).

In summary, this study provides evidence of SARS-CoV-2 infection in domestic dogs and a cat from COVID-19-positive households during quarantine at the private animal hospitals in Thailand. The role of dogs and cats in SARS-CoV-2 transmission among species or across species from animals to humans is still unclear. However, SARS-CoV-2 RNA was observed in environmental samples, thus possible transmission from contaminated areas should not be ignored. This study supports the role of the One Health approach in the mitigation and control of emerging infectious diseases, such as COVID-19, for improving human and animal health.

Table 3.9 Complete blood count and blood chemistry profiles of the SARS-CoV-2 infected dogs and cat in this study.

		Dog-A				**Dog-C**	**Dog-D**		**Cat-B**
		27 Apr 21	9 May 21	11 May 21	13 May 21	3 Jun 21	11 Jun 21		13 May 21
CBC	Dog ref.							Cat ref	
Red blood cell (10^3/ul)	5.5-8.5	6.8	6.48	6.87	6.75	6.8	7.54	5.0-10.0	8.70
Hemoglobin (g/dl)	12.0-18.0	15.4	13.8	14.5	15.3	14.4	18.0	8.0-15.0	14.8
Hematocrit (%)	37.0-55.0	45.3	44.72	47.31	46.30	48.32	55.76	24.0-45.0	48.65
MCV (fl)	60.0-77.0	66.7	69	69	69	71	74	33-55	56
MCH (pg)	19.5-24.5	22.6	21.3	21.2	22.6	21.2	23.9	12.5-17.5	17.0
MCHC (g/dl)	31.0-39.0	33.9	30.9	30.7	33.0	29.8	32.3	30.0-36.0	30.3
Platelets (10^3/ul)	200-500	413	304	384	396	163	170	250-800	265
Mean platelet volume (fl)	3.9-11.1	-	11.4	8.7	8.2	20.3	11.9	12.0-17.0	15.0
White blood cells (10^3/ul)	6.0-17.0	10.3	17.66	18.15	15.26	10.80	16.51	5.50-19.50	13.18
Lymphocytes (10^3/ul)	1.0-4.8	0.90	2.59	3.01	1.80	1.83	3.13	1.50-7.00	1.84
Monocytes (10^3/ul)	0.20-1.50	0.10	0.54	0.68	0.80	0.29	1.29	0.00-1.50	0.29
Neutrophils (10^3/ul)	3.0-12.0	8.83	14.39	14.24	12.54	0.67	11.66	2.50-14.00	10.96
Eosinophils (10^3/ul)	0.0-0.6	0.45	0.10	0.15	0.09	0.10	0.09	0.00-1.00	0.08
Basophils (10^3/ul)	0.0-0.4	0	0.04	0.07	0.04	0.00	0.01	0.00-0.20	0.00

		Dog-A				Dog-C	Dog-D		Cat-B
		27 Apr 21	9 May 21	11 May 21	13 May 21	3 Jun 21	11 Jun 21		13 May 21
Blood chemistry									
Albumin (g/dl)	2.5-44	-	3.5	3.8	3.8	-	-	2.5-3.9	-
Alkaline phosphatase (U/l)	20-150	930	1095*	1160*	1158*	-	-	6-102	-
Alanine aminotransferase (U/l)	10-118	-	154	212	257	-	-	10-100	-
Amylase (U/l)	200-1200	-	862	766	819	-	-	100-1200	-
Total bilirubin (mg/dl)/	0.1-0.6	-	0.3	0.3	0.4	-	-	0.1-0.4	-
Blood urea nitrogen (mg/dl)/	7-25	18.3	22	37	25	-	-	14-36	-
Calcium (mg/dl)/	8.6-11.8	-	10.0	10.9	11.6	-	-	8.2-10.8	-
Phosphorus (mg/dl)/	2.9-6.6	-	6.0	5.9	4.5	-	-	2.4-8.2	-
Creatinine (mg/dl)/	0.3-1.4	0.8	1.0	0.9	0.9	-	-	0.6-2.4	-
Glucose (mg/dl)/	60-110	-	46	63	32	-	-	64-170	-
Sodium (mmol/l)	138-160	-	154	146	152	-	-	145-158	-
Potassium (mmol/l)	3.7-5.8	-	5.8	4.3	3.9	-	-	3.4-5.6	-
Total protein (g/dl)	5.4-8.2	7.7	7.7	6.9	7.9	-	-	5.2-8.8	-
Globulin (g/dl)	2.3-5.2	-	2.2	3.2	4.0	-	-	2.3-5.3	-

CHAPTER 4

SARS-CoV-2 surveillance in domestic animals during the fourth wave of the COVID-19 outbreak in Thailand

This work has been published in the topic of

SARS-CoV-2 Delta variant infection in domestic dogs and cats, Thailand

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4.1 Abstract

In June–September 2021, we investigated severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infections in domestic dogs and cats (n = 225) in Bangkok and the vicinities, Thailand. SARS-CoV-2 was detected in a dog and a cat from COVID-19 positive households. Whole genome sequence analysis identified SARS-CoV-2 Delta variant of concern (B.1.617.2). Phylogenetic analysis showed that SARS-CoV-2 isolated from dog and cat were grouped into sublineage AY.30 and AY.85, respectively. Antibodies against SARS-CoV-2 could be detected in both dog (day 9) and cat (day 14) after viral RNA detection. This study raises awareness on spillover of variant of concern in domestic animals due to human-animal interface. Thus, surveillance of SARS-CoV-2 in domestic pets should be routinely conducted.

4.2 Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has been reported to infect several animal species. In Thailand, the first cases of SARS-CoV-2 infection in dogs and cats were reported in May-2021 and have raised a public health concern (Jairak *et al.*, 2022a). To date, SARS-CoV-2 spillover from humans to animals

have been reported globally in 32 countries in at least 17 animal species such as cats, dogs, mink, otter, pet ferrets, lions, tigers, pumas, snow leopards, gorillas, white-tailed deer, fishing cat, binturong, coati, hyena, lynx, hippopotamus and hamster (as of January 2022) (OIE, 2021a). In domestic dogs and cats, SARS-CoV-2 infection has been reported in several countries in America, Europe, and Asia (Newman *et al.*, 2020; Patterson *et al.*, 2020; Sailleau *et al.*, 2020; Segalés *et al.*, 2020; Sit *et al.*, 2020; Decaro *et al.*, 2021a; OIE, 2021a; Ruiz-Arrondo *et al.*, 2021).

At least five SARS-CoV-2 variants of concerns (VOC) have been classified by WHO including Alpha variant (B.1.1.7), Beta variant (B.1.351), Gamma variant (P.1), Delta variant (B.1.617.2), and Omicron (B.1.1.529) (WHO, 2022c). To date, due to the accumulate mutations of SARS-CoV-2 genome especially the mutations of the spike (S) protein which related to viral entry and binding to host cell receptor have resulted in several SARS-CoV-2 VOCs (Tang *et al.*, 2021; Tegally *et al.*, 2021). In Thailand, the delta variant (B.1.617.2) has become a predominant variant during the 4th wave of COVID-19 outbreak since July 2021 (WHO, 2021d). As of January 2022, at least 2.3 million confirmed cases with 21,959 deaths have been reported in Thailand (WHO, 2022b).

Due to human-animal interface and close contact with the owners, domestic dogs and cats have high risk of SARS-CoV-2 exposure. Spillover of the SARS-CoV-2 variant of concerns poses higher risk to domestic animals. The SARS-CoV-2, alpha variant (B.1.1.7) transmission from human to dogs and cats have been reported worldwide during early COVID-19 outbreaks (Barroso-Arevalo *et al.*, 2021; Miro *et al.*, 2021; Jairak *et al.*, 2022a). Moreover, the SARS-CoV-2, delta variant (B.1.617.2) infection in dogs emerged in the US, Spain and China, and will became more frequent scenario (Doerksen *et al.*, 2021; Fernandez-Bastit *et al.*, 2021; Kang *et al.*, 2022). During June–September 2021, the center of excellence for emerging and re-emerging infectious diseases in animals (CUEIDAs), Chulalongkorn University conducted a cross-sectional survey for SARS-CoV-2 in domestic dogs and cats in

Bangkok and the vicinities. Nasal, oral, and rectal swabs were collected from domestic dogs and cats for SARS-CoV-2 detection. We identified SARS-CoV-2 RNA from a dog and a cat from COVID-19 positive households. This study is the first to report SARS-CoV-2, B.1.617.2 (Delta variant) infection in dog and cat in Thailand.

4.3 Materials and methods

4.3.1 Sample collection from domestic dogs and cats for SARS-CoV-2.

During June–September 2021, we conducted SARS-CoV-2 survey in domestic dogs and cats living in Bangkok and the vicinities. Samples from dogs and cats were collected from participating veterinary clinics and hospitals. In total, we collected 225 samples from dogs (n = 105) and cats (n = 120) from COVID-19 positive household (n = 12) and unknown status households (n = 187) (Tables 4.1 and 4.2). We collected nasal swabs (dog; n = 8; cat; n = 16), oral swabs (dog; n = 102, cat; n = 117) and rectal swabs (dog; n = 104, cat; n = 120) from the animals by using flocked nylon swab (Copan®, California, USA). The swab samples were placed in RNA protect® (Qiagen LLC, Maryland, USA) and transported to the laboratory within 24 h. In this study, we followed up SARS-CoV-2 positive dog (n = 1) and cat (n = 1). During follow up, we collected nasal swabs (n = 9), oral swabs (n = 9), rectal swabs (n = 9) from the animals. In addition, environmental swabs, hair (n = 7), water container (n = 7), and floor (n = 7) were also collected (Table 4.3). We collected blood sample (n = 6 serum; 2 time points from a cat and 4 timepoints from a dog) from SARS-CoV-2 positive animals (Table 4.4). This study was conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand (approval No. 2031035). All methods were carried out in accordance with relevant guidelines and regulations.

4.3.2 Detection of SARS-COV-2 RNA.

Viral RNA extraction was performed by using GENTi—Automated Nucleic Acid Extraction System (GeneAll® Seoul, Korea). For the detection of SARS-CoV-2 RNA, real-time RT-PCR assays based on specific primers and probes (N2, E, and RdRp) were performed following CDC and WHO recommendations (CDC, 2020; Corman *et al.*, 2020a). In brief, a total 25 μl reaction contained 2 μl of RNA, 12.5 μl of 2X reaction buffer of the SuperScript® III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, California, USA), 1 μl of reverse transcriptase/Platinum Taq, 0.8 mM MgSO4, 0.8 μM each primer and probe and RNase-free water. Real-time RT-PCR reaction was set up at 50 °C for 15 min, followed by 95 °C for 2 min and 45 cycles of 95 °C for 15 s, and 58 °C for 30 s (E and RdRP genes) or 60 °C for 30 s (N2 gene). Samples with a Ct value of < 40 were considered positive. Due to the limitation of institute and IACUC approval, virus isolation did not perform in the study.

4.3.3 Characterization of SARS-CoV-2.

The RNA samples from the nasal swab of a cat (C27516) with SARS-CoV-2 positive collected on 15 July 2021 (Ct value 20.66) and the nasal swab of a dog (CU27791) collected on 12 September 2021 (Ct value 19.06) were subjected to whole genome ssequencing by using Oxford Nanopore. We used the ARTICS nCoV-2019 sequencing protocol V3 (LoCost) to amplify viral genome. In brief, diluted RNA (8 μl) was mixed with 2 μl of LunaScript® RT SuperMix (NEB, Ipswich, MA, USA). The cDNA synthesis was performed at 25 °C for 2 min, 55 °C for 10 min and 95 °C for 1 min. The ARTIC protocol using two pools of the SARS-CoV-2 primers for multiplex PCRs was performed by using Q5® Hot Start High-Fidelity DNA polymerase (NEB, MA, USA) with PCR reaction at 98 °C for 30 s and 35 cycles of 98 °C for 15 s and 65 °C for 5 min. After PCR amplification, library preparation was performed following the Oxford Nanopore rapid sequencing kit (SQK-RAD004) with ARTIC SARS-CoV-2 genome sequencing protocol (Freed *et al.*, 2020; Tyson *et al.*, 2020). In brief, 7.5 μl of pooled PCR products (10 μl of pool 1 and 10 μl of pool 2) was added to 2.5 μl of

fragmentation mix. Then the mixture was incubated at 30 ℃ for 1 min, 80 ℃ for 1 min, and 4 ℃ for 30 s. The mixture was cleaned by AMPure XP Bead. The mixture was loaded into Oxford Nanopore MinION device under MinKNOW version 19.12.5 software (Oxford nanopore technologies, Oxford, UK) (https://nanoporetech.com/nanopore-sequencing-data-analysis) (Baker *et al.*, 2021). After sequencing, nucleotide sequences were filtered using the sequencing summary file under the following parameters: minimum read length ≥ 500 nt and read quality ≥ 7. The qualify reads were conversed from "Fast5" into "Fastq" format by using the GPU version of the Nanopore Guppy basecaller (v3.4.4) tool. The Fastq format sequences were assembled using the genome detective program (Vilsker *et al.*, 2019) and de-novo approach with Qiagen CLC Genomics Benchwork version 20.0.4 software (QIAGEN, CA, USA) (https://digitalinsights.qiagen.com/products/qiagen-clc-mainworkbench/). Whole genome sequences of the SARS-CoV-2 were deposited into the GenBank (OK555092 and OK539641) and GISAID (EPI_ISL_5320246 and 5315539).

4.3.4 Phylogenetic analysis of SARS-CoV-2.

Whole genome sequences of SARS-CoV-2 were subjected to lineage classification by using the COVID-19 sequences of the Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) (https://cov-lineages.org/resources/pangolin.html). Phylogenetic analysis of SARS-CoV-2 was performed by comparing nucleotide sequences of 289 SARS-CoV-2 genomes available in the GISAID and GenBank database. The 5′ and 3′ untranslated regions were trimmed with at least 95% reference genome coverage (Wuhan-Hu-1) (at least 29,000 bp in length). The dataset alignment was performed by using the MAFFT FFT-NS-2 algorithm with default parameter settings (Katoh *et al.*, 2002). The maximum likelihood tree was constructed by using IQ-TREE version 2.1.3 (http://www.iqtree.org/) (Minh *et al.*, 2020) by the GTR + Γ model of nucleotide substitution (Yang, 1994), default heuristic search options, and ultrafast bootstrapping with 1000 replicates (Minh *et al.*, 2013). Tree was visualized by iTOL version 6.0 (https://itol.embl.de/) (Letunic and Bork,

2021). Lineage classification was performed by using the Pangolin tool (Rambaut *et al.*, 2021). Genetic mutation analysis of the SARS-CoV-2 was performed by comparing deduced amino acids of each gene of the viruses based on variant classifications and definitions (Kumar *et al.*, 2016; CDC, 2021).

4.3.5 Detection of SARS-CoV-2 antibodies.

The ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA kit (ID VET, Montpellier, France) was used to detect IgG antibodies against N protein of the SARS-CoV-2 virus in animal sera. We performed the ELISA test according to the manufacturer's recommendation. In brief, 25 μl of each serum sample was diluted at 1:1 ratio with the dilution buffer and added to each well. The 96-well plate was incubated at 37 °C for 45 min. The plate was washed with 300 μl of washing solution and 100 μl of N protein recombinant antigen horseradish peroxidase (HRP) conjugate was added to each well and incubated at 25 °C for 30 min. The plate was washed 3 times with 300 μl of washing solution. After washing, 100 μl of the substrate solution was added into each well and incubated at 25 °C for 20 min. Then, 100 μl of the stop solution was added. The reaction was read and recorded for the optical densities (O.D.) at 450 nm. The O.D. was calculated as the S/P percentage (S/P%). If S/P% value greater than or equal to 60% is considered positive, while sample with S/P% between 50 and 60% is considered as doubtful, and sample with S/P% < 50 is negative. The cPass SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, Jiangsu, China) was used to detect neutralizing antibodies. The assay detects SARS-CoV-2 antibodies by measurement of antibody mediated inhibition of SARS-CoV-2 RBD-ACE2 interaction. In brief, 50 μl of diluted 1:10 serum was incubated with 50 μl of HRP-conjugated RBD and incubated at 37 °C for 30 min. The 100 μl of treated serum then added to ACE2-coated ELISA plate and incubated at 37 °C for 15 min. Then the uncaptured substrate was washed out by using 260 μL of washing solution for four times. Colorimetric signal was developed by using TMB substrate at 25 °C for 15 min. Absorbance reading at 450 nm was acquired using a microplate

reader immediately after adding stop solution. The percentage inhibition was calculated. The sample with % inhibition ≥ 20% indicate the presence of SARs-CoV-2 neutralizing antibody, otherwise are negative (Michelitsch *et al.*, 2020). Pseudotype virus neutralization test was performed by using a SARS-CoV-2 lentiviral pseudotype and HEK293T expressing human ACE2. The hACE2 expressing target cells was produced by stable transduction of HEK293T cell with lentiviral vector harbouring hACE2 gene (pHAGE-EF1alphaInt-ACE2-WT, BEI Resources, VA, USA; NR-52512) and enriched by magnetic cell sorting using mouse anti-hACE2 (Sino-biological, China) and goat anti-mouse IgG microbeads with MACS LS column (Miltenyi Biotec Asia Pacific, Singapore). The SASCoV-2 lentiviral pseudotype was produced by co-transfecting plasmids pSPAX2 (Addgene, MA, USA; plasmid # 12,260), pCCGW and pHDM-SARS-CoV-2 spike (BEI Resources, VA, USA; NR-53742) into HEK293T cell. The pHDM-SARS-CoV-2 spike vector encodes the codon optimized spike gene of SARS-CoV 2 strain Wuhan-Hu-1 (GenBank NC_045512). All serum samples were heat-inactivated in a biosafety cabinet at 56 °C for 60 min and two-fold serially-diluted covering 1:20 to 1:40,960 in DMEM supplemented with 10% fetal bovine serum. The sera were incubated with 50 μL of 100 TCID50 of the SARS-CoV-2-lentiviral pseudotype at 37 °C for 1 h. Then, 50 μL of 1 × 104 HEK293T-hACE2 cell was added into the mixture and incubated at 37 °C for 48 h. A dilution at which the 50% of infection as compared to anti-SARS-CoV-2-negative serum is inhibited (IC50) was used as test cut-off. The plant-based anti-SARS-CoV-2 antibodies; H4 was used as antibody positive control (Shanmugaraj *et al.*, 2020; Tan *et al.*, 2020).

4.3.6 Ethics statement.

The Institutional Animal Care and Use Committee of the Faculty of Veterinary Science, Chulalongkorn University, Thailand approved animal study (IACUC No. 2031035). All methods were carried out in accordance with relevant guidelines and regulations. The study complies with the ARRIVE guidelines. The IACUC committee of the Faculty of Veterinary Science, Chulalongkorn University, Thailand

approved the informed consent. The informed verbal consent was obtained from all pet owners and private animal hospital staff after explaining the objectives and benefits of the study during sample collection.

4.3.7 Data availability

The authors declare that the data supporting the findings of this study are available within the article and its technical appendix files. The nucleotide sequence data have been deposited at the GenBank with accession numbers OK555092 and OK539641 and GISAID with accession numbers EPI_ISL_5320246 and EPI_ISL_5315539.

4.4 Results

4.4.1 SARS-CoV-2 infection in dogs and cats.

A cross-sectional survey for SARS-CoV-2 in dogs and cats in Bangkok and the vicinities was conducted during June to September 2021. We collected nasal, oral, and rectal swabs from 225 animals (105 dogs and 120 cats) from 199 households. Of 225 animals, 19 animals were sampled from twelve COVID-19 positive households (Table 4.1). SARS-CoV-2 detection by real-time RT-PCR assay with specific primers and probes for the N2, E and RdRp genes was performed following CDC and WHO recommendations (CDC, 2020; Corman *et al.*, 2020a). In this study, SARS-CoV-2 RNA could be detected from a cat (n = 1) and a dog (n = 1) from COVID-19 positive households. The positive swab samples were from a cat (CU27516) in Nonthaburi province in July 2021 and a dog (CU27791) in Bangkok in September 2021 (Table 4.2).

COVID-19 positive dog and cat were followed up for virological and serological investigation. A cat (CU27516) is 10 years-old, 3 kg, domestic short hair, spayed female cat. A cat was healthy and transferred to private animal hospital on 15 July 2021 (day 1) due to family members (n = 3) were COVID-19 positive and quarantined at the field hospital. All members were tested positive on 12–14 July 2021 and tested negative after 14 days. Swab (nasal, oral and rectal) and

environmental samples were collected on four occasions (day 1, 3, 7, and 10). During quarantine, a cat did not show any clinical signs. Nasal and oral swabs tested positive for SARS-CoV-2 RNA at day 1 and day 3 (Ct 20.66–34.36). Low viral RNA (high Ct Value) was detected in animal hair swabs on day 3 (Ct 36.56). All samples tested negative on day 7 and day 10 (Table 4.3 and Fig. 4.1). A dog (CU27791) is 15 years-old, 7.5 kg, Shih-Tzu breed, intact female dog. The animal was transferred to private animal hospital on 12 September 2021 due to family members (n = 4) tested positive for COVID-19 on 8–11 September 2021. Sample collection was conducted on five occasions (day 1, 3, 5, 7, and 9). SARS-CoV-2-RNA was detected in nasal and oral swabs of dogs on day 1–7 (Ct 19.06–39.87). The highest viral titers (low Ct values) were observed in nasal and oral swabs on day 3. Viral RNAs were also detected in environmental samples (hair, water container and floor) on day 3, 5, and 7 (Ct 29.87–38.13). A dog did not have fever but showed mild respiratory signs with serous nasal discharge on day 5–7 (Table 4.3 and Fig. 4.1).

In this study, we collected blood sample from SARS-CoV-2 positive animals (n = 6 sera; cat (CU27516) (n = 2 time points) and dog (CU27791) (n = 4 time points)). We tested serum samples for SARS-CoV-2 antibodies by indirect multispecies ELISA, surrogate virus neutralization test (sVNT) and pseudotyped virus neutralization (pVNT). Our result showed that a cat was positive for SARS-CoV-2 antibodies by indirect ELISA (113%-271%), sVNT (90%-95%) and pVNT (1:640) at day 14 and day 21. While a dog was positive for SARS-CoV-2 antibodies by sVNT (48.81%) at day 24 and pVNT (1:20) at day 9 and day 24 (Table 4.4).

Table 4.1 SARS-CoV-2 survey in COVID-19 households and unknown status households during COVID-19 outbreak in Thailand from June 2021 to September 2021.

Month	COVID-19 Households		Unknown status Households	
	Dog	Cat	Dog	Cat
	#Positive/#tested	#Positive/#tested	#Positive/#tested	#Positive/#tested
Jun 2021	0/1	0/8	0/54	0/41
Jul 2021	0/2	1*/5	0/2	0/5
Aug 2021	0/1	0/1	0/25	0/37
Sep 2021	1**/1	0/0	0/19	0/23
Total	**1/5**	**1/14**	**0/100**	**0/106**

*SARS-CoV-2 positive; July 15, 2021.

**SARS-CoV-2 positive; September, 12, 2021.

Table 4.2 List of COVID-19 positive households where dogs and cats were sampled and tested for SARS-CoV-2.

Household	Sample collection	Province	#of COVID-19 patients in household	Days sample collection from owner 1st positive	#SARS-CoV-2 positive swabs/# swab samples			#SARS-CoV-2 positive animals/#animals	
					Nasal swab	Oral swab	Rectal swab	Dog	Cat
1	Jun 2021	Bangkok	N/A	6 days	0/4	0/4	0/4	0/0	0/4
2	Jun 2021	Samut Prakan	2/2	2 days	0/3	0/3	0/3	0/0	0/3
3	Jun 2021	Bangkok	N/A	4 days	0/1	0/1	0/1	0/0	0/1
4	Jun 2021	Bangkok	N/A	15 days	0/1	0/1	0/1	0/1	0/0
5	Jul 2021	Bangkok	1/1	7 days	0/1	0/1	0/1	0/1	0/0
6	Jul 2021	Bangkok	1/1	15 days	0/1	0/1	0/1	0/0	0/1
7	Jul 2021	Nonthaburi	3/3	4 days	1/1*	1/1*	0/1	0/0	1/1*
8	Jul 2021	Bangkok	2/2	15 days	0/2	0/2	0/2	0/0	0/2
9	Jul 2021	Pathum Thani	5/5	10 days	0/1	0/1	0/1	0/1	0/0
10	Jul 2021	Bangkok	1/1	2 days	0/1	0/1	0/1	0/0	0/1
11	Aug 2021	Bangkok	2/2	3 days	0/2	0/2	0/2	0/1	0/1
12	Sep 2021	Bangkok	4/4	5 days	1/1**	1/1**	1/1**	1/1**	0/0
		Total			**2/19**	**2/19**	**1/19**	**1/5**	**1/14**

*SARS-CoV-2 positive from household A; July 15, 2021.

**SARS-CoV-2 positive from household B; September, 12, 2021.

Table 4.3 Result of SARS-CoV-2 detection from cat and dog swab samples by real-time-RT-PCR.

Date	Real-time RT-PCR positive (Ct value)																	
	Nasal			Oral			Rectal			Hair/body wash			Water container swab			Floor swab		
	N[a]	E[b]	RdRp[b]	N[a]	E[b]	RdRp[b]	N[a]	E[b]	RdRp[b]	N[a]	E[b]	RdRp[b]	N[a]	E[b]	RdRp[b]	N[a]	E[b]	RdRp[b]
Cat CU27516																		
15 Jul 21	+(20.66)	+(23.66)	+(28.49)	+(28.69)	+(27.28)	+(31.59)	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
17 Jul 21	+(28.70)	+(28.61)	+(31.93)	+(31.90)	+(30.99)	+(34.36)	-	-	-	-	-	+(36.56)	-	-	-	-	-	-
21 Jul 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24 Jul 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dog CU27791																		
12 Sep 21	-	+(31.98)	+(31.62)	+(38.50)	+(33.84)	+(33.15)	-	-	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
14 Sep 21	+(19.06)	+(19.86)	+(20.43)	+(27.15)	+(26.05)	+(25.48)	+(38.59)	+(35.82)	+(34.71)	+(34.61)	+(30.37)	+(29.87)	+(35.99)	+(32.07)	+(31.33)	-	+(32.92)	+(31.68)
16 Sep 21	+(27.29)	+(24.45)	+(23.17)	+(39.87)	+(34.82)	+(31.60)	-	-	-	-	+(34.36)	+(31.80)	-	+(38.13)	-	-	+(33.33)	+(31.75)
18 Sep 21	+(35.75)	+(31.82)	+(29.77)	-	+(37.38)	-	-	-	-	-	+(35.81)	+(36.31)	-	-	-	-	-	
20 Sep 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Primers and probes specific to N gene (CDC, 2020).

[b]Primers and probes specific to E and RdRp genes of SARS-CoV-2 (Corman *et al.*, 2020a).

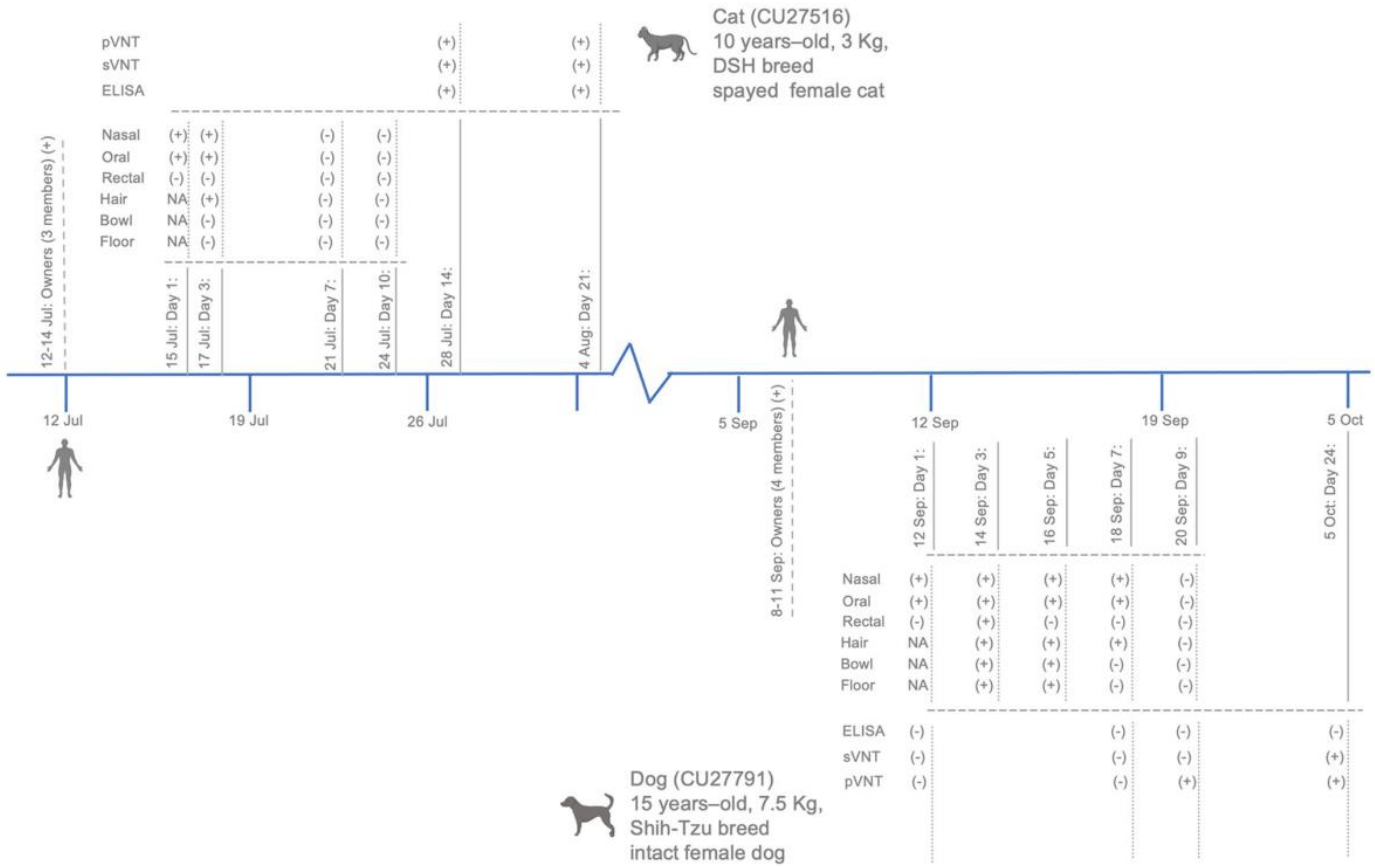

Figure 4.1 Timeline of SARS-CoV-2 detection in domestic dog and cat from COVID-19 positive households in this study.

Table 4.4 Serological test result of protein-based ELISA, sVNT and pVNT for SARS-CoV-2 antibodies in COVID-19 positive cat and dog.

ID	Host	Date	Days sample collection from animal 1st positive	Serological assay				
				ELISA*		sVNT**		pVNT***
				OD	%SP	OD	%inhibition	Titer
CU27516	Cat	28 Jul 21	14 days	1.375	113.64% (+)	0.199	90.64% (+)	640 (+)
		4 Aug 21	21 days	3.219	271.31% (+)	0.102	95.32% (+)	640 (+)
CU27791	Dog	12 Sep 21	1 day	0.056	0.63%	2.403	2.85%	<10 (-)
		18 Sep 21	7 days	0.060	0.96%	2.309	6.65%	<10 (-)
		20 Sep 21	9 days	0.157	9.06%	2.008	18.82%	20 (+)
		5 Oct 21	24 days	0.069	1.06%	1.294	48.81% (+)	20 (+)

*ELISA: ID Screen® SARS-CoV-2 Double Antigen Multi-species ELISA kit (ID VET, Montpellier, France). For the cutoff values, S/P% ≥ 60% is postive, S/P% 50–60% is suspected, and < 50% is negative.

**sVNT (Virus neutralization test): cPass SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, Jiangsu, China). The cutoff values were defined as follows; positive if % inhibition ≥ 20% is positive and negative otherwise.

***pVNT (Pseudotype Virus neutralization test): pVNT was determined against Lentiviral pseudovirus expressing spike of SARS-CoV-2 strain Wuhan-Hu-1 (Genbank NC_045512). Serum dilution that gave a 50% reduction in GFP signal (IC50) was considered positive. The titer of ≥ 10 is considered positive and negative otherwise.

4.4.2 Delta variant of SARS-CoV-2 from dog and cat in Thailand.

After SARS-CoV-2 detection, we performed whole genome sequencing from nasal swab of cat (CU27516; Ct 20.66) and dog (CU27791; Ct 19.06) by using the ARTIC multiplex PCR protocol and MinION sequencing platform (Oxford Nanopore Technologies). A total of 173,580 (CU27516) and 223,045 (CU27791) reads were archived and 85.70% (1,309 coverages) and 66.56% (2,296 coverages) were mapped to reference genome, respectively. Whole genome sequences of the viruses were 29,704 (CU27791) and 29,861 (CU27516) nucleotides with 2,296 and 1,309 coverages, respectively. Whole genome sequences of the viruses were deposited in the GenBank (OK555092 and OK539641) and GISAID (EPI_ISL_5320246 and EPI_ISL_5315539) (Table 4.5)

Phylogenetic analysis of SARS-CoV-2 was performed by comparing complete genome of SARS-CoV-2 from dog and cat in this study and 289 SARS-CoV-2 genomes available in the GISAID and GenBank database. The sequences were aligned by using the MAFFT FFT-NS-2 algorithm and phylogenetic tree was constructed by using IQ-TREE 2 applying the GTR+Γ model of nucleotide substitution with default heuristic search options and bootstrapping with 1000 replicates. Lineage classification was performed by using the Pangolin tool. Phylogenetic analysis showed that SARS-CoV-2 of dog and cat in this study clustered with human SARS-CoV-2 of B.1.617.2 (Delta variant of concern). The cat and dog SARS-CoV-2 were grouped into sublineage AY.30 (B.1.617.2.30) and AY.85 (B.1.617.2.85), respectively (Fig. 4.2). BLAST analysis of SARS-CoV-2 whole genome sequences showed that cat and dog SARS-CoV-2 possessed high nucleotide similarities to human SARS-CoV-2 of Delta variant in Aug-2021 (99.98%; COV2513/21) and Oct-2021 (99.98%; COV3783/21), respectively (Table 4.6). Characteristic mutation analysis on spike protein of SARS-CoV-2 of dog and cat showed identical mutations to those of Delta variant (Table 4.7). The mutations at the N-terminal-domain (NTD) (T19R, E156G, F157del, R158del), the receptor binding motif (RBM) (L452R, T478K), the subdomain 2 (D614G), S1 unit (P681R) and heptad

repeat 1 (D950N) were observed. It should be noted that Delta variant, AY.85 sublineage contained additional mutation at T95I on N terminal domain (NTD) and it may associate with higher viral load which promote viral transmission from human to human/animal due to close contact.

Table 4.5 Results of Nanopore sequencing of SARS-CoV-2 from dog and cat, Thailand.

Animal	Strain	GenBank accession No.	GISAID accession No.	# mapped reads	% mapped reads*	Average length (bp)	Total nt sequence	Genome size (bp)	Coverage (x)
Cat	CU 27516	OK555092	EPI_ISL_5320246	115,533	66.56	338.33	39,088,741	29,861	1,309
Dog	CU 27791	OK539641	EPI_ISL_5315539	191,132	85.70	356.97	68,229,056	29,704	2,296

*Whole-genome alignment tools; CLC Genomics Workbench version 20.0.4 (Qiagen A/S, Vedbæk, Denmark)

Table 4.6 BLAST analysis of genome sequences of SARS-CoV-2 from cat and dog with reference viruses.

Virus	GISAID/GenBank#	Location	Species	Year	Lineage	Nucleotide similarities (%)												
						WGS	ORF1ab	ORF1a	S	ORF3a	E	M	ORF6	ORF7a	ORF7b	ORF8	N	ORF10
CU27516 [a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2													
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	99.88%	99.93%	99.92%	99.79%	99.88%	100%	99.85%	100%	99.17%	100%	100%	99.60%	100%
QEUH-B80FF1	EPI_ISL_661814	UK	Human	Nov-20	B.1.1.7	99.81%	99.90%	99.89%	99.68%	99.88%	100%	99.85%	100%	99.17%	100%	99.16%	99.12%	100%
NHLS-UCTGS-7500-KRISP	EPI_ISL_696476	S. Africa	Human	Nov-20	B.1.351	99.81%	99.90%	99.88%	99.59%	99.64%	99.56%	99.70%	100%	99.17%	100%	100%	99.52%	100%
TN-CL-423-S234-R1-001	EPI_ISL_2463770	India	Human	May-21	B.1.617.2	99.92%	99.92%	99.93%	99.95%	99.88%	100%	100%	100%	99.73%	100%	99.72%	99.84%	100%
AFRIMS-COV2513-2021	MZ888556.1	Thailand	Human	Aug-21	B.1.617.2	99.98%	99.99%	99.98%	99.95%	100%	100%	100%	100%	100%	100%	100%	99.92%	100%
AFRIMS-COV3783-2021	OK626714.1	Thailand	Human	Oct-21	B.1.617.2	99.88%	99.88%	99.86%	99.92%	99.88%	99.56%	99.85%	100%	99.73%	99.23%	100%	99.76%	100%
NHSAD-21-0019	EPI_ISL_2821077	India	Lion	May-21	B.1.617.2	99.92%	99.93%	99.93%	99.95%	99.88%	100%	100%	100%	100%	100%	100%	99.84%	100%
NHSAD-21-0011	EPI_ISL_2821078	India	Lion	May-21	B.1.617.2	99.92%	99.93%	99.93%	99.95%	99.88%	100%	100%	100%	100%	100%	100%	99.84%	100%
CU27791 [b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2	99.87%	99.89%	99.86%	99.92%	99.64%	99.56%	99.85%	100%	99.73%	99.23%	100%	99.76%	100%
						WGS	ORF1ab	ORF1a	S	ORF3a	E	M	ORF6	ORF7a	ORF7b	ORF8	N	ORF10
CU27791 [b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2													
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	99.86%	99.92%	99.92%	99.82%	99.52%	99.56%	99.70%	100%	99.45%	99.23%	100%	99.68%	100%
QEUH-B80FF1	EPI_ISL_661814	UK	Human	Nov-20	B.1.1.7	99.80%	99.89%	99.89%	99.71%	99.52%	99.56%	99.70%	100%	99.45%	99.23%	99.16%	99.20%	100%
NHLS-UCTGS-7500-KRISP	EPI_ISL_696476	S. Africa	Human	Nov-20	B.1.351	99.80%	99.89%	99.87%	99.61%	99.27%	99.12%	99.55%	100%	99.45%	99.23%	100%	99.60%	100%
TN-CL-423-S234-R1-001	EPI_ISL_2463770	India	Human	May-21	B.1.617.2	99.88%	99.89%	99.88%	99.92%	99.51%	99.56%	99.85%	100%	100%	99.23%	99.72%	99.92%	100%

Virus	GISAID/GenBank#	Location	Species	Year	Lineage	Nucleotide similarities (%)												
						WGS	ORF1ab	ORF1a	S	ORF3a	E	M	ORF6	ORF7a	ORF7b	ORF8	N	ORF10
AFRIMS-COV2513-2021	MZ888556.1	Thailand	Human	Aug-21	B.1.617.2	99.86%	99.88%	99.85%	99.92%	99.64%	99.56%	99.70%	100%	99.73%	99.23%	100%	99.68%	100%
AFRIMS-COV3783-2021	OK626714.1	Thailand	Human	Oct-21	B.1.617.2	99.98%	99.98%	99.97%	100%	99.76%	100%	100%	100%	100%	100%	100%	100%	100%
NHSAD-21-0019	EPI_ISL_2821077	India	Lion	May-21	B.1.617.2	99.88%	99.89%	99.88%	99.92%	99.51%	99.56%	99.85%	100%	100%	98.99%	100%	99.92%	100%
NHSAD-21-0011	EPI_ISL_2821078	India	Lion	May-21	B.1.617.2	99.88%	99.89%	99.88%	99.92%	99.51%	99.56%	99.85%	100%	100%	98.99%	100%	99.92%	100%
CU27516 [a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2	99.87%	99.89%	99.86%	99.92%	99.64%	99.56%	99.85%	100%	99.73%	99.23%	100%	99.76%	100%

Table 4.7 Characteristic mutations of SARS-CoV-2 Delta variant from cat (AY.30) and dog (AY.85) and reference viruses.

Characteristic mutation (AY.30)														
Virus	GISAID#	Location	Species	Date	Linage	Amino acid substitution/deletion*								
						Spike gene								
						T91R	E156G	F157del	R158del	L452R	T478K	D614G	P681R	D950N
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	T	E	F	R	L	T	D	P	D
AFRIMS COV1370-2021	MZ888533	Thailand	Human	Jun-21	B.1.617.2.30	R	G	157del	158del	R	K	G	R	N
AFRIMS COV2513-2021	MZ888556	Thailand	Human	Aug-21	B.1.617.2.30	R	G	157del	158del	R	K	G	R	N
CUA21611	EPI_ISL_4488568	Thailand	Human	Jul-21	B.1.617.2.30	R	G	157del	158del	R	K	G	R	N
CU27516[a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2.30	R	G	157del	158del	R	K	G	R	N
AFRIMS COV3783-2021	OK626714	Thailand	Human	Jul-21	B.1.617.2.85	R	G	157del	158del	R	K	G	R	N
CU27791[b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2.85	R	G	157del	158del	R	K	G	R	N
						ORF1a				N				
						P309L	P1640L	H2092Y	V3718A	D63G	L139F	R203M	D377Y	R385K
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	P	P	H	V	D	L	R	D	R
AFRIMS COV1370-2021	MZ888533	Thailand	Human	Jun-21	B.1.617.2.30	L	L	Y	A	G	F	M	Y	K
AFRIMS COV2513-2021	MZ888556	Thailand	Human	Aug-21	B.1.617.2.30	L	L	Y	A	G	F	M	Y	K
CUA21611	EPI_ISL_4488568	Thailand	Human	Jul-21	B.1.617.2.30	L	L	Y	A	G	F	M	Y	K
CU27516[a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2.30	L	L	Y	A	G	F	M	Y	K
AFRIMS COV3783-2021	OK626714	Thailand	Human	Jul-21	B.1.617.2.85	P	P	H	V	G	L	M	Y	R
CU27791[b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2.85	P	P	H	V	G	L	M	Y	R

						ORF3a	M	ORF7a			ORF8		
						S26L	I82T	V82A	L116F	T120I	S84L	D119del	F120del
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	S	I	V	L	T	L	D	120del
AFRIMS COV1370-2021	MZ888533	Thailand	Human	Jun-21	B.1.617.2.30	L	T	A	F	I	L	119del	120del
AFRIMS COV2513-2021	MZ888556	Thailand	Human	Aug-21	B.1.617.2.30	L	T	A	F	I	L	119del	120del
CUA21611	EPI_ISL_4488568	Thailand	Human	Jul-21	B.1.617.2.30	L	T	A	F	I	L	119del	120del
CU27516[a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2.30	L	T	A	F	I	L	119del	120del
AFRIMS COV3783-2021	OK626714	Thailand	Human	Jul-21	B.1.617.2.85	L	T	A	L	I	L	119del	120del
CU27791[b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2.85	L	T	A	L	I	L	119del	120del

Characteristic mutation (AY.85)

Virus	GISAID#	Location	Species	Date	Linage	Amino acid substitution/deletion* Spike gene									
						T91R	T95I	E156G	F157del	R158del	L452R	T478K	D614G	P681R	D950N
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	T	T	E	F	R	L	T	D	P	D
AFRIMS COV1370-2021	MZ888533	Thailand	Human	Jun-21	B.1.617.2.30	T	T	G	157del	158del	R	K	G	R	N
AFRIMS COV2513-2021	MZ888556	Thailand	Human	Aug-21	B.1.617.2.30	R	T	G	157del	158del	R	K	G	R	N
CUA21611	EPI_ISL_4488568	Thailand	Human	Jul-21	B.1.617.2.30	R	T	G	157del	158del	R	K	G	R	N
CU27516[a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2.30	R	T	G	157del	158del	R	K	G	R	N
AFRIMS COV3783-2021	OK626714	Thailand	Human	Jul-21	B.1.617.2.85	R	I	G	157del	158del	R	K	G	R	N
CU27791[b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2.85	R	I	G	157del	158del	R	K	G	R	N

						ORF1a						N			
						E148G	A1306S	P2046L	P2287S	V2930L	T3255I	D63G	R203M	G215C	D377Y
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	E	A	P	P	V	T	D	R	G	D
AFRIMS COV1370-2021	MZ888533	Thailand	Human	Jun-21	B.1.617.2.30	E	A	P	P	V	T	G	M	G	Y
AFRIMS COV2513-2021	MZ888556	Thailand	Human	Aug-21	B.1.617.2.30	E	A	P	P	V	T	G	M	G	Y
CUA21611	EPI_ISL_4488568	Thailand	Human	Jul-21	B.1.617.2.30	E	A	P	P	V	T	G	M	G	Y
CU27516[a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2.30	E	A	P	P	V	T	G	M	G	Y
AFRIMS COV3783-2021	OK626714	Thailand	Human	Jul-21	B.1.617.2.85	G	S	L	S	L	I	G	M	G	Y
CU27791[b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2.85	E	S	L	S	L	I	G	M	G	Y
						ORF3a		E	M	ORF7a		ORF7b	ORF8		
						S26L	L140F	V62F	I82T	V82A	T120I	T40I	S84L	D119del	F120del
Wuhan-Hu-1	NC_045512.2	China	Human	Dec-19	B	S	L	V	I	V	T	T	L	D	F
AFRIMS COV1370-2021	MZ888533	Thailand	Human	Jun-21	B.1.617.2.30	L	L	V	T	A	I	T	L	119del	120del
AFRIMS COV2513-2021	MZ888556	Thailand	Human	Aug-21	B.1.617.2.30	L	L	V	T	A	I	T	L	119del	120del
CUA21611	EPI_ISL_4488568	Thailand	Human	Jul-21	B.1.617.2.30	L	L	V	T	A	I	T	L	119del	120del
CU27516[a]	EPI_ISL_5320246	Thailand	Cat	Jul-21	B.1.617.2.30	L	L	V	T	A	I	T	L	119del	120del
AFRIMS COV3783-2021	OK626714	Thailand	Human	Jul-21	B.1.617.2.85	L	F	F	T	A	I	I	L	119del	120del
CU27791[b]	EPI_ISL_5315539	Thailand	Dog	Sep-21	B.1.617.2.85	L	F	F	T	A	I	I	L	119del	120del

[a]Cat-SARS-CoV-2 characterized in this study. [b]Dog-SARS-CoV-2 characterized in this study.

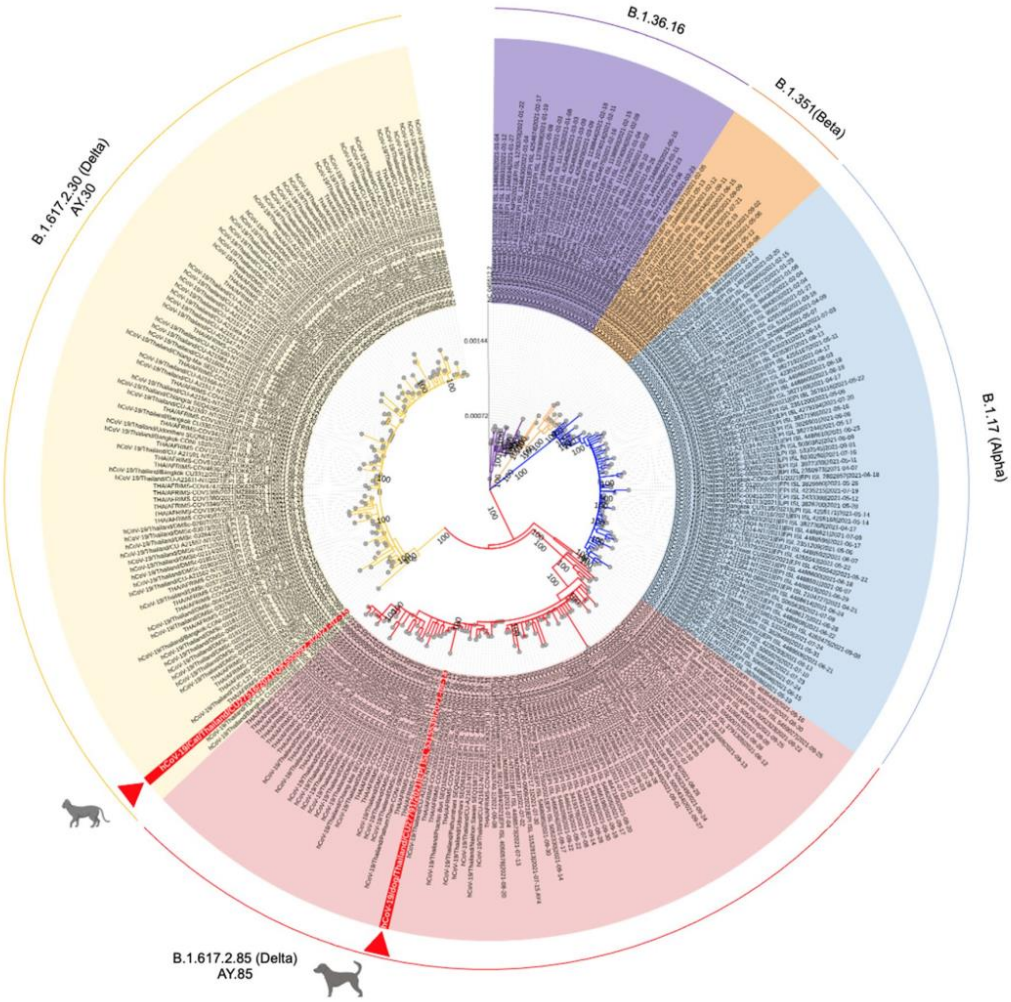

Figure 4.2 The maximum likelihood tree of SARS-CoV-2 from dog, cat and human from Thailand.

Tree was constructed by using IQ-TREE version 2.1.3 (http://www.iqtree.org/) using the GTR + Γ model of nucleotide substitution, default heuristic search options, and ultrafast bootstrapping with 1000 replicates. Tree was visualized by iTOL version 6.0 (https://itol.embl.de/). Colors indicate PANGO lineages including purple (B.1.36.16), orange (B.1.351; Beta), blue (B.1.1.7; Alpha), pink (B.1.617.2.85; Delta AY.85) and yellow (B.1.617.2.30; Delta AY.30). Red arrows indicate SARS-CoV-2 from dog and cat in this study.

4.5 Discussion

In this study, we conducted a cross-sectional survey in dogs and cats in private animal hospitals from June–September 2021. During investigation period, we identified SARS-CoV-2 delta variant of concern (B.1.617.2) in dogs and cats with the occurrence of 20.0% (1/5) in dogs and 7.1% (1/14) in cats (16.7%; 2 from 12 COVID-19 households). Notably, the occurrence of SARS-CoV-2 may relate to timing of sample collection, health condition of animals and close contact with the owners (Calvet *et al.*, 2021; Jairak *et al.*, 2022a; Jairak *et al.*, 2022b). The B.1.617.2 lineage was a predominant lineage of the recent 4th wave of COVID-19 outbreaks in Thailand (WHO, 2021d). Comparing to the previous study in Thailand, SARS-CoV-2 infection in dogs and cats in April to May 2021, dogs and cats were infected with the prevalence of 8.6% (3/35) in dogs and 11.1% (1/9) in cats (23.5%; 4 from 17 COVID-19 households) and SARS-CoV-2 of the Alpha variant (B.1.1.7 lineage) was identified (Jairak *et al.*, 2022a). Notably, SARS-CoV-2 Delta variant (B.1.617.2) infection in dogs was first reported in Kansas, USA and Barcelona, Spain in mid-2021 (Doerksen *et al.*, 2021; Fernandez-Bastit *et al.*, 2021). In cats, SARS-CoV-2 Delta variant (B.1.617.2) infection in cats was reported in Harbin, China in September 2021 (Kang *et al.*, 2022). Moreover, SARS-CoV-2 Delta variant infection have been reported in Asiatic lions in zoological park in India and white-tail deer in Canada (Mishra *et al.*, 2021; Karikalan *et al.*, 2022).

Virological test for SARS-CoV-2 showed that viral RNA could be detected from nasal and oral swabs of a cat and a dog at day 1 (4–5 days after the owner reported COVID-19 positive). It is interesting to note that SARS-CoV-2 RNA could also be detected in animal hair of cat at day 3 and environmental samples (hair, water container and floor) of dog at day 3 and day 7. Similarly, in previous reports, SARS-CoV-2 RNA could be found in environmental samples e.g. water container and cage floor of domestic pet as well as dust and air samples in mink farms (Chaintoutis *et al.*, 2021a; Jairak *et al.*, 2022a). Serological test for SARS-CoV-2 antibodies in this

study showed that a cat developed SARS-CoV-2 antibodies since day 14 (by ELISA, sVNT and pVNT) after SARS-CoV-2 RNA detection. While a dog was positive for SARS-CoV-2 antibodies at day 9 (by pVNT) and day 24 (by sVNT and pVNT). Our findings in agreement with previous studies that dogs and cats can develop antibodies against SARS-CoV-2 as early as 7 to 14 days post infection (Barrs *et al.*, 2020; Hamer *et al.*, 2020; Patterson *et al.*, 2020). For example, our previous studies showed that SARS-CoV-2 antibodies could be detected from dogs at day 11–23 and from cats at day 6 after SARS-CoV-2 RNA detection. Notably, control dog and cat sera and pre-COVID-19 sera have been tested in our previous report (Jairak *et al.*, 2022a). However, the timing for serum sample collection when the animal might have been exposed to COVID-19 owners could influence the antibodies titer and the discrepancy between the result of ELISA, sVNT and pVNT.

Phylogenetic analysis showed that the cat and dog SARS-CoV-2 were grouped into Delta variant of concern (B.1.617.2) sublineage AY.30 (B.1.617.2.30) and AY.85 (B.1.617.2.85), respectively. Both sublineages are the predominant sublineages of Delta variant in the 4th wave of COVID-19 outbreaks in Thailand with the prevalence of 71.0% and 77.0%, respectively (Pangolin lineage; https://cov lineages.org/lineage_list.html). Unlike previous studies, SARS-CoV-2 Delta variant, sublineage AY.3 was responsible for COVID-19 in dog in the US and sublineage AY.43 in dog in Spain (Doerksen *et al.*, 2021; Fernandez-Bastit *et al.*, 2021). Genetic mutation analysis of the cat and dog SARS-CoV-2 showed that all mutations agreed with those of delta variant Delta variant of concern (B.1.617.2) including the mutations at the N-terminal-domain (NTD), the receptor binding motif (RBM), the subdomain 2, S1 unit and heptad repeat 1. Since SARS-CoV-2, B.1.617.2 lineage has higher transmissibility rates than the B.1.1.7 viruses, thus, mutations on spike protein in relation to transmissibility or host adaptation of animal SARS-CoV-2 needed further investigation (Dhar *et al.*, 2021). A previous study reported role of mutations on spike gene may not in relation to hostadapted mutations (McAloose *et al.*, 2020; Dhar *et*

al., 2021). Notably, unlike previous study, M1227L on S gene was not observed in our SARS-CoV-2 from dog. It speculated that this point mutation may relate to S protein destabilization and transmission from human-to-dog (Broer *et al.*, 2006; Doerksen *et al.*, 2021).

In conclusion, results from phylogenetic and mutation analysis suggested that the virus infecting dog and cat in this study originated from a local outbreak cluster of Delta variant AY.30 and AY.85. Notably, AY.30 and AY.85 sub-lineages are predominantly found in Thailand. In Thailand, most veterinary clinics and hospitals have followed COVID-19 management guideline provided by the department of livestock development, Thailand. Since most SARS-CoV-2 infected animals are asymptomatic or less symptomatic. History of close contact between owners and domestic animals in COVID-19 positive household is crucial for veterinary practitioners to monitor SARS-CoV-2 infection in domestic animals. This study is the first to report SARS-CoV-2 Delta variant infection in domestic dogs and cats in Thailand. Our finding supports routine surveillance of SARS-CoV-2 in domestic animals and raises more awareness on frequent spillover of variant of concerns due to high human-animal interface.

CHAPTER 5

SARS-CoV-2 surveillance in wildlife in Thailand

This work is in the manuscript preparation in the topic of

SARS-CoV-2 surveillance in wildlife in Thailand and the infection in captive lions and a tiger

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5.1 Abstract

Due to the emergence of SARS-CoV-2 in wildlife species, such as bats and pangolins, the surveillance of SARS-CoV-2 in several wildlife species has been recommended. This study aimed to conduct the SARS-CoV-2 surveillance in wildlife species in captive and free-ranging habitats including zoos, wildlife breeding centers, and natural areas where humans and wildlife interface in Thailand, during pre and post-COVID-19 (from 2017 to 2023). Swabs and sera were collected from various mammals, including primates, cervids, felids, mustelids, viverrids, elephants, wild boars, kangaroos, sloths, bears, banteng, and pangolin. The samples were tested for SARS-CoV-2 RNA and antibodies by the real-time RT-PCR and sVNT, respectively. Out of 364 animals, only seven lions from Zoo A in July 2022 tested positive for immunity to SARS-CoV-2, while all others tested negative. In Zoo A, we conducted a SARS-CoV-2 retrospective investigation, and tissue samples were obtained from a tiger that had died from heart and renal failure, with an exposure history to a COVID-19-positive animal caretaker since December 2021. SARS-CoV-2 RNA was found in the lung tissue of a tiger, and it belonged to SARS-CoV-2 Delta VOCs (B.1.617.2), which was the predominant variant during the outbreak in Thailand at that time. It is noted

that the seven seropositive felids were likely to have immunity for 8 months following SARS-CoV-2 infection by the same staff at a tiger event. This study also reported cases of human-to-animal transmission of SARS-CoV-2 in tiger and lions in Thailand. The passive and active surveillance, as well as COVID-19 human exposure history monitoring in wildlife species, should be concerned and continued. This information will help identify the factors contributing to the transmission and evolution of SARS-CoV-2 as part of a comprehensive One Health approach.

5.2 Introduction

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) causing the coronavirus disease 2019 (COVID-19) emerged in December 2019 (Gorbalenya *et al.*, 2020; Lu *et al.*, 2020; WHO, 2020b). The first outbreak of COVID-19 was reported in the seafood or wildlife market in China (Bagcchi, 2020; Zhou *et al.*, 2020). This pandemic disease rapidly spread among the human population worldwide, causing significant impacts on health and the economy (Xiong *et al.*, 2020; Mohapatra *et al.*, 2022). It has been documented that SARS-CoV-2 is a zoonotic viral pathogen with bat origins (Zhou *et al.*, 2020; Wacharapluesadee *et al.*, 2021). There have been several reports of host-adapted mutations of SARS-CoV-2 as a result of the virus circulation among the animal population and spill back to humans (Bashor *et al.*, 2021; Oude Munnink *et al.*, 2021; Pickering *et al.*, 2022; Sun *et al.*, 2022; Tan *et al.*, 2022). As of December 2022, natural SARS-CoV-2 infection has been reported in 24 different animal species worldwide (cats, dogs, mink, otters, ferrets, lions, tigers, pumas, snow leopards, Indian leopards, gorillas, white-tailed deer, fishing cats, binturongs, South American coati, spotted hyena, Eurasian lynx, Canada lynx, hippopotamus, hamster, mule deer, giant anteater, West Indian manatee, black-tailed marmoset) (OIE, 2022; Qiu *et al.*, 2023). Due to the high mutation rate, at least 5 SARS-CoV-2 variants of concern (VOCs) have been defined by WHO, including Alpha VOCs (B.117), Beta VOCs (B.1.351), Gamma VOCs (P.1), Delta VOCs (B.1.617.2) and Omicron VOCs (B.1.1529)

(WHO, 2022c). It has been reported that SARS-CoV-2 Omicron variant infections in domestic animals and wildlife species are less frequent and less severe compared to other SARS-CoV-2 variants (Martins *et al.*, 2022b; Sanchez-Morales *et al.*, 2022; van Doremalen *et al.*, 2022; Virtanen *et al.*, 2022; Zhang *et al.*, 2022; Lyoo *et al.*, 2023). However, deer and mink are highly susceptible to the Omicron variant, and there have been concerned about virus circulation and their potential role as natural reservoirs (Domanska-Blicharz *et al.*, 2022; Vandegrift *et al.*, 2022). Several studies suggested that SARS-CoV-2 surveillance should be focused more on wild animals to assess the risk of spillover, the potential for widespread transmission, and the evolution of the virus (Sun *et al.*, 2022; Italiya *et al.*, 2023). Zoo settings or rehabilitation centers with close interaction between wildlife species and humans are suitable for SARS-CoV-2 surveillance (Santini and Edwards, 2020). SARS-CoV-2 detection in free-ranging wildlife, such as deer, with a high prevalence, the emergence of deer-specific variant, and the potential of spillover back to humans emphasized the importance of monitoring SARS-CoV-2 in wildlife to prevent disease outbreaks in both humans and animals (Pickering *et al.*, 2022). This study aimed to determine SARS-CoV-2 infection in captive and free-ranging wildlife across various settings, including zoos, wildlife breeding centers, and natural areas where humans and wildlife interact in Thailand during pre-COVID-19 and post-COVID-19 (2017-2023). Our findings could be used to provide recommendations for effective mitigation strategies for preventing animals at risk and SARS-CoV-2 spillover. The information from this study will help identify the factors contributing to the transmission and evolution of SARS-CoV-2 as part of a comprehensive One Health approach.

5.3 Materials and Methods

5.3.1 Sample collection from wildlife for SARS-CoV-2 investigation

In this study, we conducted a retrospective and cross-sectional SARS-CoV-2 investigation in free-ranging and captive wildlife in Thailand during 2017-2023.

For a retrospective study, we obtained tissue samples of captive primates, felids, viverrids, mustelids, and cervids collected during 2017-2019. Tissue samples were acquired from the Genetic Resource Bank of ZPOT. In free-ranging wildlife, we obtained oral and rectal swabs from macaques during the population control and disease surveillance program of the Department of National Parks, Wildlife and Plant Conservation during 2017-2019. The samples included oral and rectal swabs from animals. The swab samples were preserved at -20°C.

For a cross-sectional study, we conducted a SARS-CoV-2 survey in captive and free-ranging wildlife from 2020 to 2023. For captive wildlife, we collected samples from the animals from zoos and wildlife breeding facilities. The animals were restrained by veterinarian officers for routine health check-ups, disease diagnosis treatment purposes, and population control programs. We collected nasal, oral, and rectal swabs and stored at -20°C.

5.3.2 Detection of SARS-COV-2 RNA

For swab samples, the samples were vigorously vortexed for 15 seconds before aliquoting the supernatant for RNA extraction. For tissue and fecal samples, 2 mg of tissue samples or feces was mixed with 2 ml of PBS using Qiagen Powerlyzer homogenizer machine (Qiagen, Hilden, Germany) for homogenization. For RNA extraction, 200 μl of supernatant from swabs, feces, and tissue samples were used for RNA extraction by using GENTi-Automated Nucleic Acid Extraction System (GeneAll® Seoul, Korea).

For SARS-CoV-2 RNA detection, the viral RNA was detected by using real-time RT-PCR assays with primers and probes specific to E and RdRp specific to SARS-CoV-2 following WHO recommendations (Corman *et al.*, 2020a). In brief, a 25 μl reaction contained 2 μl of RNA, 12.5 μl of 2X reaction buffer of the SuperScript® III

Platinum® One-Step Quantitative RT-PCR System (Invitrogen, California, USA), 1 μl of reverse transcriptase, 0.8 mM MgSO4, 0.8 μM each primer and probe and RNase-free water. Real-time RT-PCR reaction was set up at 50 °C for 15 min, followed by 95 °C for 2 min and 45 cycles of 95 °C for 15 s, and 58 °C for 30 s. Samples with a Ct value of < 40 were considered positive.

5.3.3 Characterization of SARS-CoV-2

The SARS-CoV-2 positive RNA by real-time RT-PCR was subjected to whole genome sequencing by using the Oxford Nanopore sequencing technique. We applied the ARTICS nCoV-2019 sequencing protocol V3 (LoCost) to amplify the viral genome (Quick, 2020). In brief, diluted RNA (8 μl) was mixed with 2 μl of LunaScript® RT SuperMix (NEB, Ipswich, MA, USA). The cDNA synthesis was performed at 25 °C for 2 min, 55 °C for 10 min, and 95 °C for 1 min. The ARTIC protocol using two pools of the SARS-CoV-2 primers for multiplex PCRs was performed by using Q5® Hot Start High-Fidelity DNA polymerase (NEB, MA, USA) with PCR reaction at 98 °C for 30 s and 35 cycles of 98 °C for 15 s and 65 °C for 5 min. After PCR amplification, library preparation was performed by the Oxford Nanopore rapid sequencing kit (SQK-RAD004) according to the manufacturer's protocol (Oxford Nanopore Technologies®, Oxford, UK). In brief, 7.5 μl amplicon from two pools of SARS-CoV-2 multiplex PCR primers was added to 2.5 μl of fragmentation mix. Then the mixture was incubated at 30 °C for 1 min, 80 °C for 1 min, and 4 °C for 30 s. The mixture was cleaned by AMPure XP Bead and was loaded into an Oxford Nanopore MinION device operating through MinKNOW version 19.12.5 software (Oxford Nanopore Technologies®, Oxford, UK) (https://nanoporetech.com/nanopore-sequencing-data-analysis) (Baker *et al.*, 2021).

After sequencing, the qualified nucleotide sequences with minimum read length ≥ 500 nt and read quality ≥ 7 were conversed from "Fast5" into "Fastq" format by using the GPU version of the Nanopore Guppy basecaller (v3.4.4) tool. The Fastq format sequences were assembled using the de-novo approach with Qiagen

CLC Genomics Benchwork version 20.0.4 software (QIAGEN, CA, USA) (https://digitalinsights.qiagen.com/products/qiagen-clc-mainworkbench/). The lineage of SARS-CoV-2 was identified and assigned by using the web application of the Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLIN) (https://cov-lineages.org/resources/pangolin.html) and the Nextclade application (https://clades.nextstrain.org/) (Aksamentov *et al.*, 2021; O'Toole *et al.*, 2021).

Phylogenetic analysis was performed by comparing nucleotide sequences of at least 100 isolates of SARS-CoV-2 genomes from Thailand available in the GISAID database. The SARS-CoV-2 nucleotide sequence dataset was aligned using MAFFT version 7 web application with default parameters (https://mafft.cbrc.jp/alignment/server/) (Katoh *et al.*, 2009). The aligned sequence dataset was subjected to phylogeny analysis using MEGA X software based on the neighbor-joining method. Genetic mutation analysis of the SARS-CoV-2 was performed by comparing amino acid substitutions with different variants of SARS-CoV-2 by MEGA X software.

5.3.4 SARS-CoV-2 antibody detection

The sera samples were tested for antibody-mediate inhibition of SARS-CoV-2 RBD-ACE2 interaction by using the cPass™ SARS-CoV-2 neutralization antibody detection kit based on the surrogate virus neutralization test (sVNT) technique (GenScript Biotech, Jiangsu, China). Briefly, 10 μl of serum samples, as well as positive and negative controls, were diluted as 1:10 in sample dilution buffer. After 1:1 mixing with HRP-RBD working solution, the mixture was incubated at 37°C for 30 min. Then, 100 μl of samples were loaded into each well (pre-coated hACE2) and incubated at 37°C for 15 min. The plate was washed for 4 times. Then 100 μl of TMB substrate was added into each well and incubated at room temperature for 15 min. Then the 50 μl of stop solution was added. The optical density (OD) was immediately measured at 450 nm using a microplate reader. The percentage inhibition (% inhibition) was calculated. The sample with ≥30% inhibition and <30% inhibition was considered positive and negative, respectively.

5.3.5 Ethics

Sample collection from wildlife was conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Science, Chulalongkorn University, Thailand (approval No. 2031050). The permission for wildlife sample usage was approved by the Department of National Parks, Wildlife and Plant Conservation (DNP) (No. 0907.4/24957). The Permission for sample collection from zoo animals was approved by the Zoological Park Organization of Thailand (ZPOT) (No.1108/1036).

5.4 Results

5.4.1 Sample collection from wildlfie

During 2017-2023, we acquired and collected samples for the SARS-CoV-2 survey in wildlife species. The samples were obtained from 364 animals, including 85 animals from 2017 to 2019 (Pre COVID-19) and 279 animals from 2020 to 2023 (Post COVID-19) from 10 provinces of Thailand (Fig 5.1) Wildlife species, including primates, cervids, felids, mustelids, viverrids, elephants, wild boars, kangaroos, sloths, bears, banteng, and pangolin (Table 5.1 and 5.2).

Table 5.1 Number of wildlife samples collected during pre-COVID-19 and post-COVID-19, by provinces of Thailand.

Province	Region	No. of animals		Total
		Pre COVID-19 (2017-2019)	Post COVID-19 (2020-2023)	
Chiang Mai	North	0	11	**11**
Lop Buri	Central	10	0	**10**
Samut Sakhon	Central	0	2	**2**
Prachuap Khiri Khan	Central	25	0	**25**
Chon Buri	East	50	185	**235**
Ubon Ratchathani	North-East	0	51	**51**
Surin	North-East	0	10	**10**
Nakhon Ratchasima	North-East	0	5	**5**
Songkhla	South	0	14	**15**
Surat Thani	South	0	1	**1**
Total		**85**	**279**	**364**

Table 5.2 List of wildlife samples acquired/collected in this study, by group/family of wildlife.

Animal group/family	Pre-COVID-19 (2017-2019)	Post COVID-19 (2020-2023)	Total
Primates	55	116	**171**
Cervidae	8	56	**64**
Felidae	8	33	**41**
Musteloidae	6	36	**42**
Viverroidea	8	8	**16**
Elephantidae	0	10	**10**
Suidae	0	7	**7**
Macropodidae	0	5	**5**
Choloepodidae	0	4	**4**
Ursidae	0	2	**2**
Bovidae	0	1	**1**
Manidae	0	1	**1**
Total	**85**	**279**	**364**

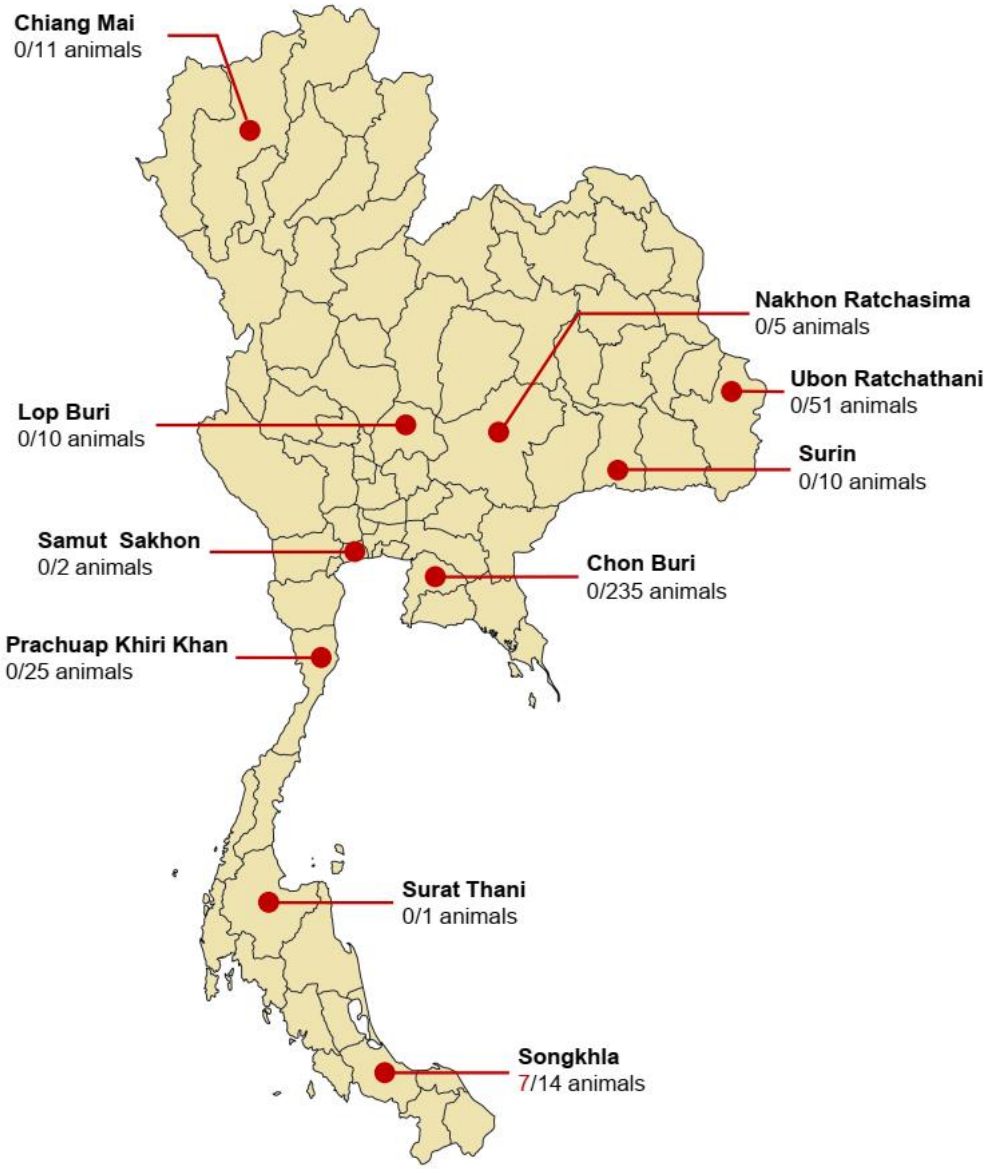

Figure 5.1 Map of Thailand and 10 provinces where the samples (n=364) were acquired /collected in this study.

5.4.2 SARS-CoV-2 RNA detection

For pre-COVID-19 (2017-2019), we acquired oral and rectal swab samples (n=90) from 45 macaques and tissue samples (n=80) from 40 different wildlife species including primates, cervids, felids, mustelids, and viverrids. The samples were tested for SARS-CoV-2 by using real-time RT-PCR, and our result showed that all samples (n=170) of swabs and tissue samples were negative for SARS-CoV-2. For post-COVID-19 (2020-2023), we collected oral and rectal swabs (n=424) and feces (n=23) from various wildlife species. Our results showed that all samples (n=447) were negative for SARSS-CoV-2 (Table 5.3).

Table 5.3 The result of SARS-CoV-2 detection in wildlife samples during pre-COVID-19 (2017-2019) and post-COVID-19 (2020-2023).

Animal group	Pre-COVID-19 (2017-2019)			Post COVID-19 (2020-2023)		
	Swab	Tissue	Serum	Swab	Feces	Serum
Primates	0/90	0/20	0/0	0/232	0/0	0/0
Cervidae	0/0	0/16	0/0	0/6	0/23	0/30
Felidae	0/0	0/16	0/0	0/48	0/0	[a]7/9
Musteloidae	0/0	0/12	0/0	0/72	0/0	0/0
Viverroidea	0/0	0/16	0/0	0/16	0/0	0/0
Elephantidae	0/0	0/0	0/0	0/20	0/0	0/0
Suidae	0/0	0/0	0/0	0/14	0/0	0/0
Macropodidae	0/0	0/0	0/0	0/0	0/0	0/5
Choloepodidae	0/0	0/0	0/0	0/8	0/0	0/0
Ursidae	0/0	0/0	0/0	0/4	0/0	0/0
Bovidae	0/0	0/0	0/0	0/2	0/0	0/0
Manidae	0/0	0/0	0/0	0/2	0/0	0/0
Total	**0/90**	**0/80**	**0/0**	**0/424**	**23**	**7/44**

[a] 7 lions from Zoo A with SARS-CoV-2 seropositive by sVNT.

5.4.3 SARS-CoV-2 antibody detection

In this study, 44 sera from wildlife, including felids, cervids, and kangaroos, collected between 2020 and 2023, were tested for antibodies to SARS-CoV-2 by sVNT assay. Our result showed that 7 sera were positive for SARS-CoV-2 antibodies. The positive samples were collected from white and African lions in Zoo A in July 2022. All positive serum samples demonstrated significant antibody levels against SARS-CoV-2 by sVNT assay with % inhibition ranging from 47.47% to 97.15% (cut off =30% of inhibition). On the other hand, the serum samples from wildlife in the same zoo (fishing cats) were negative (Table 5.4 and 5.5).

Table 5.4 The result of antibodies against SARS-CoV-2 by using sVNT assay from 3 different zoos during post COVID-19.

Place	Animal species	Animal group	sVNT[a] result (positive/tested)
Zoo A	White lion	*Felidae*	**3**/3
	African lion	*Felidae*	**4**/4
	Fishing cat	*Felidae*	0/2
Zoo B	Barasingha	*Cervidae*	0/1
	Sambar deer	*Cervidae*	0/1
Zoo C	Sambar deer	*Cervidae*	0/5
	Eld's deer	*Cervidae*	0/5
	Scimitar horn oryx	*Cervidae*	0/2
	Red kangaroo	*Macropodidae*	0/5
	Hog deer	*Cervidae*	0/5
	Barasingha	*Cervidae*	0/5
	Sika deer	*Cervidae*	0/6
	Total		**7/44**

Table 5.5 Details of wildlife sera and antibodies against SARS-CoV-2 by using sVNT assay in this study.

Place	Animal species	Animal group	Collected date	sVNT[a] % inhibition 1st	sVNT[a] % inhibition 2nd
Zoo A	White lion	*Felidae*	July 2022	**85.67% (+)**	**87.29% (+)**
	White lion	*Felidae*	July 2022	**40.06% (+)**	**47.47% (+)**
	White lion	*Felidae*	July 2022	**97.23% (+)**	**97.15% (+)**
	African lion	*Felidae*	July 2022	**45.47% (+)**	**69.59% (+)**
	African lion	*Felidae*	July 2022	**95.19% (+)**	**88.87% (+)**
	African lion	*Felidae*	July 2022	**67.93% (+)**	**58.55% (+)**
	African lion	*Felidae*	July 2022	**61.97% (+)**	**66.20% (+)**
	Fishing cat	*Felidae*	July 2022	-30.18%	0.81%
	Fishing cat	*Felidae*	July 2022	-28.60%	14.66%
Zoo B	Barasingha	*Cervidae*	May 2022	-5.41%	ND
	Sambar deer	*Cervidae*	May 2022	-13.82%	ND
Zoo C	Sambar deer	*Cervidae*	November 2021	-15.06%	ND
	Sambar deer	*Cervidae*	November 2021	-20.98%	ND
	Sambar deer	*Cervidae*	January 2022	-26.52%	ND
	Sambar deer	*Cervidae*	February 2022	-20.38%	ND
	Eld's deer	*Cervidae*	January 2022	7.58%	ND
	Eld's deer	*Cervidae*	January 2022	3.93%	ND
	Eld's deer	*Cervidae*	January 2022	-0.32%	ND
	Eld's deer	*Cervidae*	January 2022	1.99%	ND
	Eld's deer	*Cervidae*	January 2022	9.47%	ND
	Scimitar horn oryx	*Cervidae*	January 2022	19.23%	6.61%
	Scimitar horn oryx	*Cervidae*	January 2022	12.15%	ND
	Red kangaroo	*Macropodidae*	January 2022	13.08%	ND
	Red kangaroo	*Macropodidae*	January 2022	-8.32%	ND
	Red kangaroo	*Macropodidae*	January 2022	-10.91%	ND
	Red kangaroo	*Macropodidae*	January 2022	-9.61%	ND
	Red kangaroo	*Macropodidae*	January 2022	-21.30%	ND
	Hog deer	*Cervidae*	May 2022	2.08%	ND
	Hog deer	*Cervidae*	May 2022	-5.18%	ND
	Hog deer	*Cervidae*	May 2022	-11.65%	ND
	Hog deer	*Cervidae*	May 2022	-5.45%	ND

Hog deer	*Cervidae*	May 2022	-14.74%	ND
Barasingha	*Cervidae*	June 2022	-16.27%	ND
Barasingha	*Cervidae*	June 2022	-16.31%	ND
Barasingha	*Cervidae*	June 2022	-8.83%	ND
Barasingha	*Cervidae*	June 2022	-11.32%	ND
Barasingha	*Cervidae*	June 2022	-2.73%	ND
Sika deer	*Cervidae*	June 2022	-0.09%	ND
Sika deer	*Cervidae*	June 2022	-11.00%	ND
Sika deer	*Cervidae*	June 2022	-11.32%	ND
Sika deer	*Cervidae*	June 2022	-24.82%	ND
Sika deer	*Cervidae*	June 2022	-13.63%	ND
Sika deer	*Cervidae*	June 2022	-6.24%	ND

[a]VNT (Virus neutralization): The cPass™ SARS-CoV-2 Neutralization Antibody Detection Kit (GenScript Biotech, Jiangsu, China). The cut off value, if % inhibition ≥ 30% is positive, <30% is negative.

ND: Not done the test

5.4.4 Retrospective study of SARS-CoV-2 in animals in Zoo A

In this study, we conducted a retrospective investigation for SARS-CoV-2 infection in Zoo A. Since our result showed SARS-CoV-2 seropositivity in 7 lions in Zoo A during July 2022, this finding suggested the SARS-CoV-2 infection in wildlife and possibly humans/workers in the zoo. Based on our retrospective investigation by record review, interviewed veterinarians, zoo staff, and animal caretakers, it has been confirmed that staff A, feline species animal caretaker, was confirmed COVID-19 positive on November 25, 2021. During that period (November 2021), the record showed female tiger, age 16, died on December 3, 2021. The animal showed clinical signs of dyspnea, anorexia, and lethargy for 10 days (clinical signs started on November 23, 2021). There were 4 feline caretakers, who had been contacted with the animals, and only staff A tested positive for COVID-19 on November 25, 2021. The zoo veterinarians performed an examination on the tiger carcass. The gross pathology showed severe congestion and edema of the lungs, moderate cardiomegaly with myocardium hypertrophy, and contracted granular and congestion of kidneys. Histopathological examination showed hemosiderophages (cells associated with heart failure) and proteinaceous material accumulation in the form of the hyaline membrane were found in the alveolar space. The myocardium of the heart displayed fatty infiltration, and the interstitial tissue of the kidneys contained mononuclear inflammatory cells. Based on postmortem and histopathological examination, the tiger was diagnosed with nephritis, viral pneumonia, and chronic heart failure (Fig 5.2, 5.3, and 5.4).

Since the animal had been contacted with SARS-CoV-2 positive caretaker (Staff A) and died with respiratory symptoms. We acquired the frozen organ specimens of the tiger, including the trachea, paratracheal lymph node, lung, and intestine, to test for SARS-CoV-2 RNA by real-time RT-PCR. Our result showed that SARS-CoV-2 RNA could be detected from lung tissue but negative from other tissue samples (trachea, lymph node, and intestine), The lung tissue sample (CU28108L)

showed weak positive results for SARS-CoV-2 specific to N, E, and RdRp genes with Ct values of 33.89, 29.25 and 31.55, respectively (Fig 5.5).

In this study, due to the very low viral quantity of the positive lung tissue of the tiger (CU28108L), the partial SARS-CoV-2 sequences were acquired with gaps of 17,474 nucleotides. For the spike gene, there were 3,258 nucleotides (gaps 564 nucleotides), or 85.24%, when compared to the reference spike gene EPI_ISL_402124. The classification of SARS-CoV-2 from tiger based on partial S gene sequences by using Nextclade assigned the virus at SARS-CoV-2 Delta variants.

The phylogenetic tree of the spike gene of SARS-CoV-2 from the tiger (CU28108L) and 135 human SARS-CoV-2 spike gene isolates from Thailand, including lineage B.1.1.7 (Alpha VOCs), B.1.351 (Beta VOCs), B.1.617.2 (Delta VOCs), and B.1.1.519.2 (Omicron VOCs) available in GISAID database was generated. Our results showed that the spike gene of tiger SARS-CoV-2 (CU28108L) belongs to the sublineage AY.59 of Delta VOCs (B.1.617.2.59) (Fig. 5.5.). The genetic analysis of CU28108L spike gene mutation revealed amino acid substitutions similar to those of Delta variants.

For example, there were mutations in the S1 subunit (D614G, P681R), N-terminal domain (NTD) (G142D, E156G, F157del, R158del), receptor-binding domain (RBD) (L452R, T478K), and heptapeptide repeat sequence 2 (HR2) (D1163G) (Table 5.6).

Figure 5.2 The tiger carcass was thin, scoring a 2 on the body condition rating related to chronic disease.

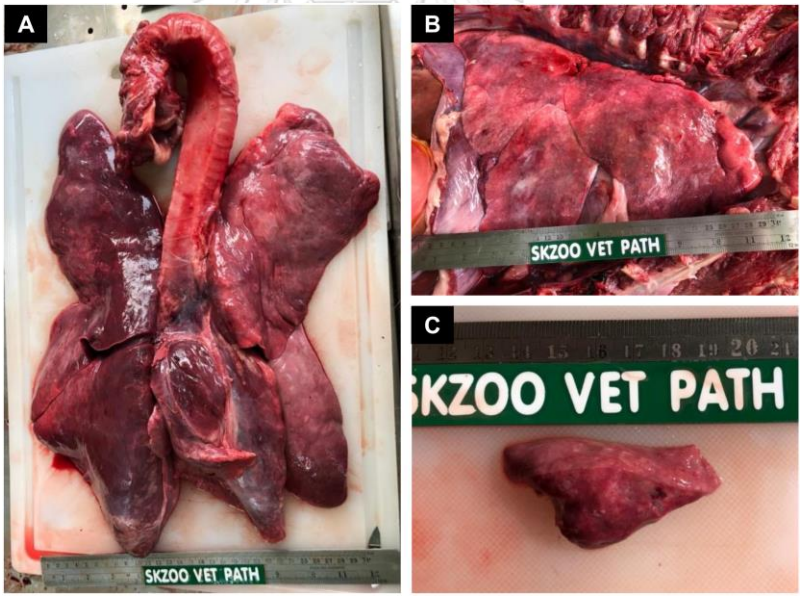

Figure 5.3 Gross lesions of the tiger's lung with positive SARS-CoV-2 RNA (CU28108L).

A; Ventral view of whole lungs showed lung congestion and edema, generalized dark red, and multifocal consolidations throughout the lungs. **B and C;** Diffused consolidation and congestion areas presented in all lobes of the right lung (cranial, middle and caudal lobe) and lung surface, respectively.

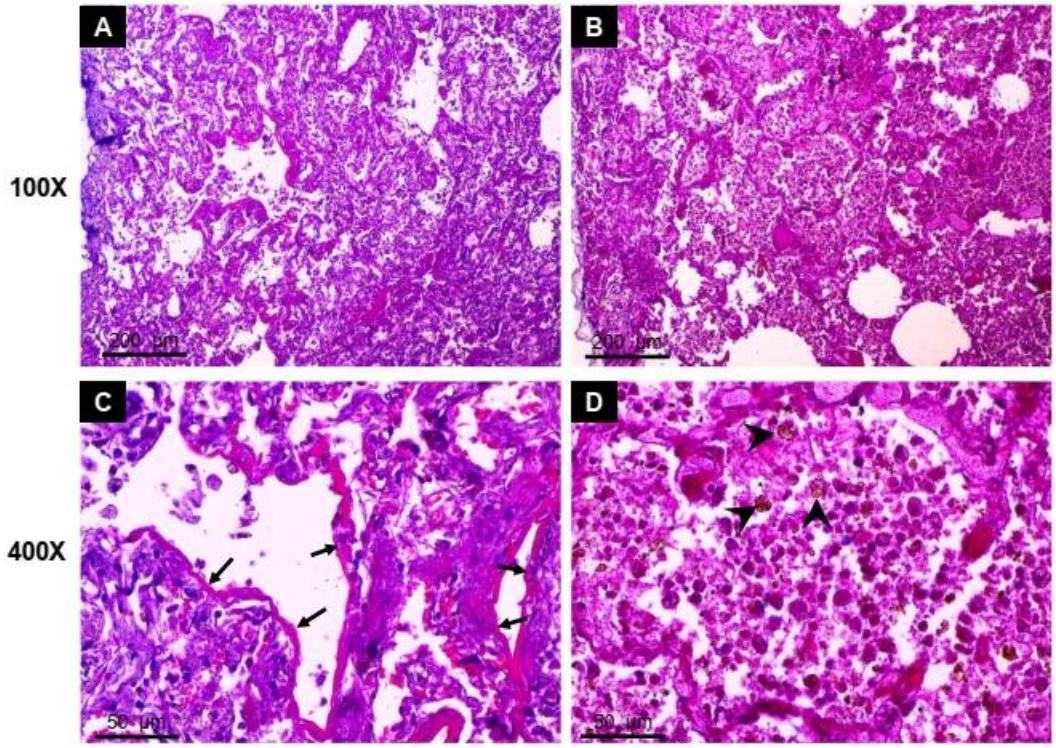

Figure 5.4 Histopathological findings from lung tissue of SARS-CoV-2 positive tiger by hematoxylin-eosin staining.

A; Severe acute interstitial pneumonia infiltrated by mononuclear inflammatory cells with thickened alveolar wall. **B; and C** Multifocal proteinaceous material accumulation in the alveolar spaces, forming hyaline membranes (arrows). **D;** The alveolar spaces filled with mixed inflammatory cells including neutrophils, mononuclear inflammatory cells and hemosiderin-laden macrophages (Heart failure cells) (arrows).

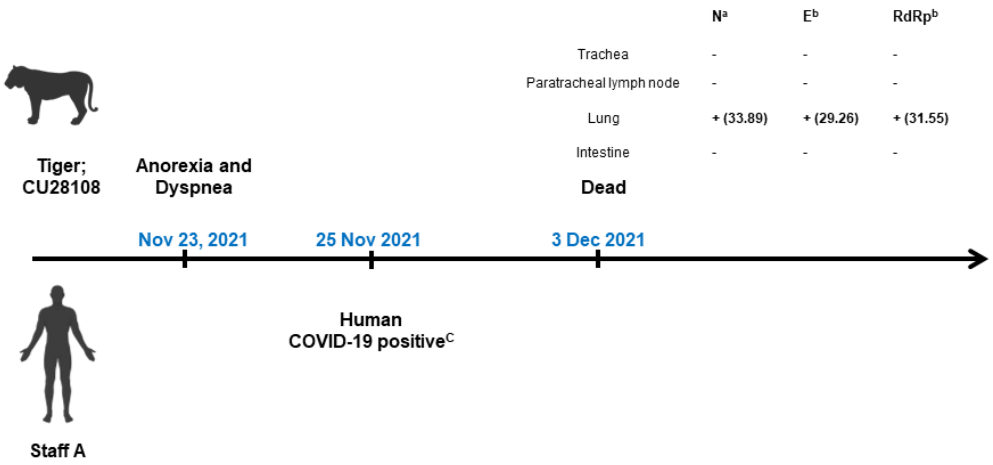

Figure 5.5 Timeline of SARS-CoV-2 in tiger (CU28108) and staff A in the Zoo.

[a]Tiger was confirmed positive COVID-19 from samples collected at December 3, 2021 by real-time RT-PCR with primers and probes specific to N gene (CDC, 2020), E gene, and RdRp gene of SARS-CoV-2 (Corman *et al.*, 2020a)

[b] Staff A was confirmed COVID-19 positive at November 25, 2021 by real-time RT-PCR test at local authorized hospital.

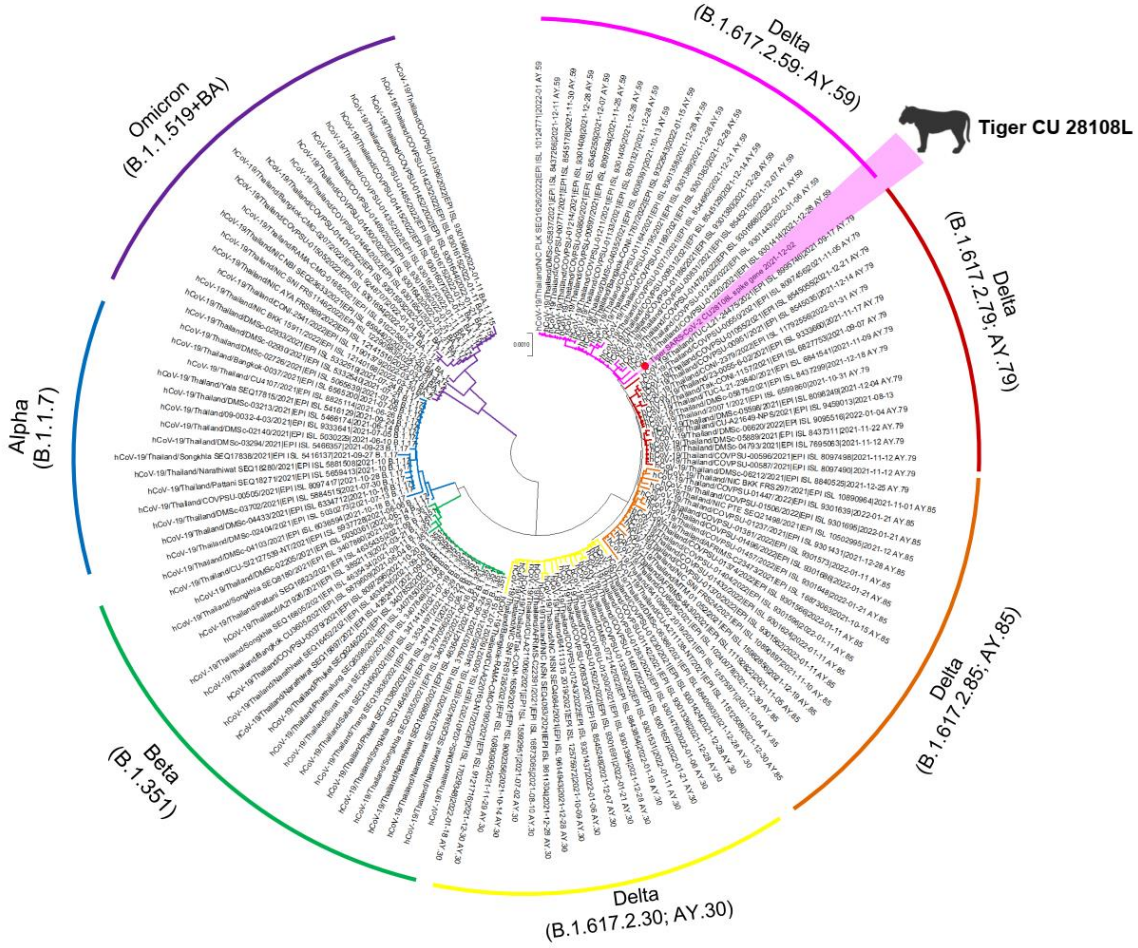

Figure 5.6 The phylogenetic tree of the spike gene of SARS-CoV-2.

The tiger SARS-CoV-2 clustered with the Delta variant, subvariant AY.59. Phylogenetic tree was generated by using MEGA X software and applying a neighbor-joining algorithm with 1000 bootstrap replications.

Table 5.6 Genetic analysis of amino acid substitutions of the spike protein of tiger SARS-CoV-2 comparing with the reference viruses from different VOCs in Thailand.

					Amino acid substitution/deletion*													
Virus	**GISAID#**	**Location**	**Date**	**Linage**	18	19	24	25	26	27	69	70	80	95	142	146	156	157
Human_WIV04	EPI_ISL_402124	China	2019-12-30	B	L	T	L	P	P	A	H	V	D	T	G	H	E	F
Human_SEQ17838	EPI_ISL_5416137	Thailand	2021-09-27	B.1.1.7	L	T	L	P	P	A	**del**	**del**	D	T	G	H	E	F
Human_SEQ14649	EPI_ISL_3797056	Thailand	2021-08-17	B.1.351	**F**	T	L	P	P	A	H	V	**A**	T	G	H	E	F
Human_DMSc_06386	EPI_ISL_8840693	Thailand	2021-12-28	B.1.617.2.30	L	**R**	L	P	P	A	H	V	D	T	G	H	E	**del**
Human_DMSc-04333	EPI_ISL_6334611	Thailand	2021-10-19	B.1.617.2.59	L	**R**	L	P	P	A	H	V	D	T	G	H	**G**	**del**
Human_TUC-L21-24475	EPI_ISL_8995746	Thailand	2021-09-17	B.1.617.2.79	L	**R**	L	P	P	A	H	V	D	T	**D**	**L**	**G**	**del**
Human_CU6696	EPI_ISL_10240078	Thailand	2021-12-30	B.1.617.2.85	L	**R**	L	P	P	A	H	V	D	**I**	**D**	H	E	**del**
Human_CONI-2541	EPI_ISL_11901379	Thailand	2022-3-21	B.1.1.519.BA.2	L	**I**	**S**	**del**	**del**	**del**	H	V	D	T	**D**	H	E	F
Tiger_ CU28108L		Thailand	2021-12-03		**n**	**n**	**n**	**n**	**n**	**n**	H	V	D	T	**D**	H	**G**	**del**
Virus	**GISAID#**	**Location**	**Date**	**Linage**	158	213	215	222	241	242	243	262	339	371	373	375	376	405
Human_WIV04	EPI_ISL_402124	China	2019-12-30	B	R	V	D	A	L	L	A	A	G	S	S	S	T	D
Human_SEQ17838	EPI_ISL_5416137	Thailand	2021-09-27	B.1.1.7	R	V	D	A	L	L	A	**V**	G	S	S	S	T	D
Human_SEQ14649	EPI_ISL_3797056	Thailand	2021-08-17	B.1.351	R	V	**G**	A	**del**	**del**	**del**	A	G	S	S	S	T	D
Human_DMSc_06386	EPI_ISL_8840693	Thailand	2021-12-28	B.1.617.2.30	**del**	V	D	A	L	L	A	A	G	S	S	S	T	D
Human_DMSc-04333	EPI_ISL_6334611	Thailand	2021-10-19	B.1.617.2.59	**del**	V	D	**V**	L	L	A	A	G	S	S	S	T	D
Human_TUC-L21-24475	EPI_ISL_8995746	Thailand	2021-09-17	B.1.617.2.79	**del**	V	D	A	L	**del**	**del**	A	G	S	S	S	T	D
Human_CU6696	EPI_ISL_10240078	Thailand	2021-12-30	B.1.617.2.85	**del**	V	D	A	L	L	A	A	G	S	S	S	T	D
Human_CONI-2541	EPI_ISL_11901379	Thailand	2022-3-21	B.1.1.519.BA.2	R	**G**	D	A	L	L	A	A	**D**	**F**	**P**	**F**	**A**	**N**
Tiger_ CU28108L		Thailand	2021-12-03		**del**	V	D	A	**n**	L	A	**n**	G	S	S	S	T	D
Virus	**GISAID#**	**Location**	**Date**	**Linage**	408	417	440	452	478	484	493	498	501	505	570	614	655	679
Human_WIV04	EPI_ISL_402124	China	2019-12-30	B	R	K	N	L	T	E	Q	Q	N	Y	A	D	H	N
Human_SEQ17838	EPI_ISL_5416137	Thailand	2021-09-27	B.1.1.7	R	K	N	L	T	E	Q	Q	**Y**	Y	**D**	**G**	**Y**	N

Human_SEQ14649	EPI_ISL_3797056	Thailand	2021-08-17	B.1.351	R	N	N	L	T	K	Q	Q	Y	Y	A	G	H	N
Human_DMSc_06386	EPI_ISL_8840693	Thailand	2021-12-28	B.1.617.2.30	R	K	N	R	K	E	Q	Q	N	Y	A	G	H	N
Human_DMSc-04333	EPI_ISL_6334611	Thailand	2021-10-19	B.1.617.2.59	R	K	N	R	K	E	Q	Q	N	Y	A	G	H	N
Human_TUC-L21-24475	EPI_ISL_8995746	Thailand	2021-09-17	B.1.617.2.79	R	K	N	R	K	E	Q	Q	N	Y	A	G	H	N
Human_CU6696	EPI_ISL_10240078	Thailand	2021-12-30	B.1.617.2.85	R	K	N	R	K	E	Q	Q	N	Y	A	G	H	N
Human_CONI-2541	EPI_ISL_11901379	Thailand	2022-3-21	B.1.1.519.BA.2	S	N	K	L	K	A	R	R	Y	H	A	G	Y	K
Tiger_ CU28108L		Thailand	2021-12-03		R	K	N	R	K	E	Q	Q	N	Y	n	G	H	N
Virus	**GISAID#**	**Location**	**Date**	**Linage**	**681**	**701**	**716**	**764**	**796**	**936**	**950**	**954**	**969**	**982**	**1118**	**1163**	**1199**	
Human_WIV04	EPI_ISL_402124	China	2019-12-30	B	P	A	T	N	D	D	D	Q	N	S	D	D	D	
Human_SEQ17838	EPI_ISL_5416137	Thailand	2021-09-27	B.1.1.7	H	A	I	N	D	D	D	Q	N	A	H	D	Y	
Human_SEQ14649	EPI_ISL_3797056	Thailand	2021-08-17	B.1.351	P	V	T	N	D	D	D	Q	N	S	D	D	D	
Human_DMSc_06386	EPI_ISL_8840693	Thailand	2021-12-28	B.1.617.2.30	R	A	T	N	D	D	N	Q	N	S	D	D	D	
Human_DMSc-04333	EPI_ISL_6334611	Thailand	2021-10-19	B.1.617.2.59	R	A	T	N	D	D	N	Q	N	S	D	D	D	
Human_TUC-L21-24475	EPI_ISL_8995746	Thailand	2021-09-17	B.1.617.2.79	R	A	T	N	D	D	N	Q	N	S	D	D	D	
Human_CU6696	EPI_ISL_10240078	Thailand	2021-12-30	B.1.617.2.85	R	A	T	N	D	Y	N	Q	N	S	D	D	D	
Human_CONI-2541	EPI_ISL_11901379	Thailand	2022-3-21	B.1.1.519.BA.2	H	A	T	K	Y	D	D	H	K	S	D	D	D	
Tiger_ CU28108L		Thailand	2021-12-03		R	A	T	N	D	D	D	Q	N	S	D	G	D	

5.5 Discussion

In this study, we conducted SARS-CoV-2 surveillance in wildlife species, including primates, cervids, felids, mustelids, viverrids, elephants, wild boars, kangaroos, sloths, bears, banteng, and pangolin from zoos, wildlife breeding center, and wildlife natural areas during pre-COVID-19 (2017-2019) and post-COVID-19 (2020-2023). All samples from wildlife tested negative for SARS-CoV-2 RNA. In detail, during the pre-COVID-19 period (2017-2019), tissue samples or swab samples from 85 animals were negative for SARS-CoV-2. During the post-COVID-19 period (2020-2023), all swab samples from 279 animals were negative. On the other hand, the seroprevalence of SARS-CoV-2 in wildlife species was 15.91% (7/44). Our result showed higher seroprevalence when compared to the previous study of SARS-CoV-2 in wildlife in Croatia, where the seroprevalence of SARS-CoV-2 in free-ranging wildlife and zoo animals was 2.81% (15/533). Notably, 15 animals tested positive for SARS-CoV-2 nucleocapsid (N) based ELISA, but all samples were negative by the sVNT assay (Jemersic *et al.*, 2021). In our study, serum samples were collected from captive wildlife in zoos and the seroprevalence was 15.91% (7/44). Compared to the report from the USA, the serosurvey in free-ranging white-tailed deer showed 37.04% (20/54) positivity (Palermo *et al.*, 2022). It was speculated that human-contaminated sources spillover was a potential cause of the high incidence of infection in deer in the USA (Hale *et al.*, 2022; Palermo *et al.*, 2022). In this study, wildlife species in zoos and wildlife breeding facilities had a lower incidence of SARS-CoV-2 infection, likely due to the limited exposure to outside sources and strict animal management practices that minimize intraspecies transmission. The animal caretakers were identified as the main potential source of spillover.

The positive serum samples (n=7) were collected from lions in Zoo A in July 2022. The average level of SARS-CoV-2 neutralizing antibodies (%inhibition) was 70.50% with ranges between 40.06% and 97.23% (30% cut-off). Unfortunately, the swab samples were not collected from these animals due to the sVNT assay being

performed several months after blood sample collection. According to the history taken, none of the seropositive lions displayed any clinical symptoms between 2021 and 2023.

Due to the seropositive of SARS-CoV-2 found in Zoo A, we conducted a retrospective outbreak investigation for SARS-CoV-2 in wildlife in Zoo A. The information from a retrospective outbreak investigation reported that an animal caretaker of felids species, staff A, was confirmed COVID-19 positive on November 25, 2021. In the same zoo, a 16-year-old female tiger developed dyspnea and anorexia on November 23, 2021. Then, on December 3, 2021, the animal died of respiratory distress. Gross and histopathological findings showed lesions of heart failure, pneumonia, and nephritis. The histopathological finding revealed severe subacute diffuse intestinal pneumonia with alveolar edema, alveolar and interstitial inflammatory cells infiltration, and hyaline membrane, which are frequent lesions of COVID-19 patients and symptomatic animal cases (Molenaar *et al.*, 2020; Batah and Fabro, 2021; Palmer *et al.*, 2021). We acquired the tissue of the tiger, including the trachea, paratracheal lymph node, lung, and intestine, and then tested for SARS-CoV-2 by real-time RT-PCR. SARS-CoV-2 RNA was detected in the lung tissue, while other tissue samples were negative.

Our observation was similar to other previous studies that SARS-CoV-2 infection in cats, tigers, lions, and a leopard could be fatal and was associated with other health problems (Carpenter *et al.*, 2021; Mishra *et al.*, 2021; Madhusoodanan, 2022; Mahajan *et al.*, 2022). A SARS-CoV-2 infection in experimental cats showed severe lung lesions without any respiratory symptoms but viral RNA could not be detected in the lungs (Barroso-Arevalo *et al.*, 2023). Another study reported that some SARS-CoV-2 positive tigers and lions exhibited mild symptoms with recovery within four weeks (McAloose *et al.*, 2020; Fernandez-Bellon *et al.*, 2021; Mitchell *et al.*, 2021; Karikalan *et al.*, 2022; Koeppel *et al.*, 2022).

The partial spike gene of SARS-CoV-2 characterized in this study belongs to the SARS-CoV-2 Delta variant (B.1.617.2), which was the predominant variant causing the COVID-19 outbreak in Thailand during late 2021. It is noted that the animal was kept in a separate enclosure and never shown in a public animal exhibition or allowed to share the exhibition area with other tigers. Thus, the SARS-CoV-2 infection in this tiger was suggested as the spillover event from an animal caretaker to the animal. Our finding was similar to a previous study that SARS-CoV-2 cases in zoo animals resulted from exposure to COVID-19 caretakers (McAloose *et al.*, 2020; Bartlett *et al.*, 2021; Fernández-Bellon *et al.*, 2021; Nagy *et al.*, 2022; Wang *et al.*, 2022).

Genetic analysis of the partial SARS-CoV-2 spike gene determined the characteristic amino acid alterations of the SARS-CoV-2 Delta variant (B.1.617.2). The amino acid mutations included N-terminal domain (NTD) (G142D, E156G, F157del, R158del), receptor-binding domain (RBD) (L452R, T478K) and the S1 subunit (D614G, P681R) (Tian *et al.*, 2021; Kumar *et al.*, 2022). Notably, mutation of D1163G residue was observed in the spike protein of SARS-CoV-2 in this study. D1163G residue locates in the heptapeptide repeat sequence 2 (HR2) region of the S2 subunit that relates to membrane transfusion between the virus and host cell for viral entry (Fan *et al.*, 2020; Wang *et al.*, 2020b). However, the mutation of spike D1163G does not enhance the infectivity and immune escape (Ruiz-Rodriguez *et al.*, 2021).

The possible source of SARS-CoV-2 infection in seven seropositive lions could be the animal caretaker (staff A) since December 2021, as same as the tiger or the other sources. In case of infection by staff A since December 2021, the lions that had seropositive in Jul 2022, might have long-duration immunity to SARS-CoV-2 of 8 months. There were several reports of SARS-CoV-2 in animals with long-duration immunity. For example, an experimental study in Syrian hamsters showed antibodies against SARS-CoV-2 longer than 6 months after viral inoculation (Field *et al.*, 2022). In another study in Barcelona Zoo, 4 COVID-19 positive lions had SARS-CoV-2

neutralizing antibodies at least 4 months after respiratory symptoms onset (Fernandez-Bellon *et al.*, 2021). The study in dogs and cats showed that neutralizing antibodies against the virus were present for more than 3 months (Kuhlmeier *et al.*, 2023). Interestingly, in the study in the USA, white-tailed deer possessed SARS-CoV-2 neutralizing antibodies for at least 13 months after natural infection (Hamer *et al.*, 2022).

In Zoo A, there were 41 wildlife of felid species including lions (n=9), 7 tigers (n=7), leopard cats (n=7), fishing cats (n=7), flatted-headed cats (n=6) and leopards (n=2). All animals did not show any clinical signs at the time of blood sample collection, including 7 SARS-CoV-2 positive lions. Most captive tigers and lions with SARS-CoV-2 reported cases displayed mild respiratory symptoms and recovered within four weeks (McAloose *et al.*, 2020; Fernandez-Bellon *et al.*, 2021; Mitchell *et al.*, 2021; Karikalan *et al.*, 2022; Koeppel *et al.*, 2022). However, several reports indicated that wildlife species with SARS-CoV-2 infection did not show clinical symptoms. An active SARS-CoV-2 surveillance in captive wildlife species in a zoo in the USA, including binturongs, snow leopards, tigers, and coatis, showed SARS-CoV-2 RNA positive in feces and nasal swabs, but all animals appeared healthy (Allender *et al.*, 2022). Four seropositive tigers from a SARS-CoV-2 serosurvey in Thailand in 2020–2021 showed no signs of respiratory illness during the study period (Sangkachai *et al.*, 2022). The naturally and experimentally infected white-tailed deer with SARS-CoV-2 did not exhibit any respiratory distress (APHIS, 2021; Palmer *et al.*, 2021).

In conclusion, the study, during the pre-covid-19 period, could not be found any active SARS-CoV-2 infection in wildlife species. During the post-COVID-19 period, SARS-CoV-2 infection could not be detected in free-ranging wildlife but it was found in captive wildlife (Zoo A), including a tiger and seven lions. From phylogenetic tree and amino acid substitution analysis, the tiger's partial spike gene of SARS-CoV-2 belonged to the SARS-CoV-2 Delta variant. Genetic analysis of the tiger’s virus and animal history suggested that these positive animals were infected with SARS-CoV-2

by COVID-19 animal caretaker spillover. SARS-Cov-2 infection in lions was asymptomatic, but it may have related to the tiger's fatality with other health problems. Our findings encourage passive and active surveillance, as well as monitoring in animals with a history of COVID-19 human case exposure, in wildlife species, which can provide information on viral evolution and early detection of the SARS-CoV-2.

CHAPTER 6

Conclusions and Recommendations

The coronavirus disease of 2019 (COVID-19) is a severe respiratory disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus was first identified in Wuhan, China, in late December 2019, associated with seafood or live animal markets (WHO, 2020b). SARS-CoV-2 has rapidly spread among human populations worldwide, resulting in a pandemic with a significant impact on public health, economy, and society (Xiong *et al.*, 2020; Mohapatra *et al.*, 2022). Several studies reported that SARS-CoV-2 is a zoonotic pathogen and bats are likely a natural reservoir for the virus (Wacharapluesadee *et al.*, 2021; Wu *et al.*, 2022). Since the COVID-19 pandemic outbreak, several SARS-CoV-2 infections in animals have been reported due to spillover from humans to animals (Barroso-Arevalo *et al.*, 2021; Fernandez-Bastit *et al.*, 2021; Jairak *et al.*, 2022a). However, SARS-CoV-2 reverse transmissions from animals to humans were also reported in farmed minks, pet hamsters, deer, and cats (Hammer *et al.*, 2021; Oude Munnink *et al.*, 2021; Yen *et al.*, 2022). It has been speculated that host-adapted mutations of SARS-CoV-2 due to the virus circulation in animal populations and then spill-back to humans could contributed to novel SARS-CoV-2 variants (Bashor *et al.*, 2021; Oude Munnink *et al.*, 2021; Pickering *et al.*, 2022; Sun *et al.*, 2022; Tan *et al.*, 2022). Thus, SARS-CoV-2 surveillance in domestic animals and wildlife species can provide beneficial information for public health awareness and risk communication regarding disease status, occurrence, and species at risk for SARS-CoV-2 infection. To date, Thailand has experienced at least five waves of COVID-19 outbreaks in humans. This dissertation explored retrospective and cross-sectional SARS-CoV-2 surveillances in domestic animals and wildlife species in Thailand from 2017 to 2023. The results from retrospective and cross-sectional SARS-CoV-2 surveillance in domestic animals and wildlife species were provided in chapters 2, 3, 4, and 5.

In Chapter 2, we conducted a survey of SARS-CoV-2 in domestic dogs and cats in Samut Sakhon Province, the epicenter of the second wave of the COVID-19 outbreak in Thailand in February 2021. SARS-CoV-2 RNA and antibodies were tested in swab samples and sera from dogs and cats living in high-risk settings, including field hospitals and COVID-19 positive households. A total of 523 swab samples (nasal, oral, and rectal swabs) and 159 sera were obtained and examined for SARS-CoV-2 infection in dogs (n = 83) and cats (n = 93). Although, all swab samples tested negative for SARS-CoV-2 RNA by real-time RT-PCR, 3.14% (5/159) animals tested positive for anti-N-IgG antibodies against SARS-CoV-2 by indirect multispecies ELISA. This study demonstrated possible SARS-CoV-2 exposure in domestic animals living in high-risk areas during the second wave of the COVID-19 outbreak in Thailand.

In Chapter 3, we conducted active surveillance focusing on dogs and cats from COVID-19 households during the third wave of the SARS-CoV-2 outbreak in Thailand. From April to May 2021, we confirmed 3 dogs and 1 cat with SARS-CoV-2 infection among 44 animals from 17 COVID-19 households. SARS-CoV-2 RNA was detected from nasal, oral, rectal, and environmental swabs of dog-A, cat-B, dog-C, and dog-D. These animals tested positive for SARS-CoV-2 RNA from 4 to 30 days following the pet owners' COVID-19 confirmation. Only dog-A displayed mild clinical signs, while the others remained asymptomatic. Neutralizing antibodies against SARS-CoV-2 were detected in all four positive animals. We obtained three SARS-CoV-2 whole genome sequences from two dogs and one cat and then conducted the phylogenetic and genomic mutation analysis. SARS-CoV-2 from all three animals belonged to the SARS-CoV-2 Alpha variant (B.1.1.7 lineage), the predominant lineage of the third wave of the COVID-19 outbreak in Thailand. This study provides evidence of SARS-CoV-2 infection in domestic dogs and a cat from COVID-19-positive households. Notably, SARS-CoV-2 RNA was also observed in environmental samples, thus possible transmission from contaminated areas should not be ignored. Public

awareness of SARS-CoV-2 infection in pet dogs and cats in close contact with COVID-19 patients should be raised.

In Chapter 4, we conducted a cross-sectional SARS-CoV-2 survey in domestic dogs and cats in Bangkok and vicinities during the 4th wave of the COVID-19 outbreak in Thailand. From June to September 2021, nasal, oral, and rectal swabs were collected from 225 animals, including dogs and cats, from both COVID-19 positive and unknown-status households. Real-time RT-PCR and serological tests indicated SARS-CoV-2 infections in a dog and a cat from COVID-1 9 positive households. Phylogenetic analysis demonstrated that SARS-CoV-2 from a dog and a cat belonged to the SARS-CoV-2 Delta variant (B.1.617.2) sub-lineage AY.30 and AY.85, respectively. Antibodies against SARS-CoV-2 could also be detected in both the dog (day 9) and the cat (day 14) after viral RNA detection. This study raises awareness on the spillover of variants of concern in domestic animals due to the human-animal interface and suggests that SARS-CoV-2 surveillance in domestic pets should be routinely conducted.

In Chapter 5, we conducted SARS-CoV-2 surveillance in wildlife species in both captive and free-ranging habitats in Thailand, including zoos, wildlife breeding centers, and natural areas where humans and wildlife interface during the pre and post-COVID-19 (from 2017 to 2023). SARS-CoV-2 RNA and antibodies were tested in swabs and sera from primates, cervids, felids, mustelids, viverrids, elephants, wild boars, kangaroos, sloths, bears, banteng, and pangolin. From 364 animals, all samples tested negative for SARS-CoV-2 RNA by real-time RT-PCR. While 15.91% (7/44) tested positive for antibodies against SARS-CoV-2 by sVNT assay. The positive serum samples were collected from lions from Zoo A in July 2022. From a retrospective outbreak investigation in Zoo A, we acquired tissue samples from a tiger with a history of exposure to a confirmed COVID-19 positive animal caretaker in December 2021. Our result showed SARS-CoV-2 positive in the lung tissue of a tiger. Phylogenetic analysis of the spike gene of the virus showed that the virus belonged to the SARS-CoV-2

Delta variant (B.1.617.2). It is noted that the seropositive felids were likely to have immunity for 8 months since viral infection in December 2021. The findings of this study provided SARS-CoV-2 dynamic information in wildlife populations and the potential for spillover transmission, as well as the importance of ongoing active and passive surveillance of SARS-CoV-2 in wildlife species especially in animals with a history of COVID-19 human exposure.

From the results of this study, we formulated the recommendations for SARS-CoV-2 in domestic animals and wildlife, as in the following:

Domestic animals

- Monitoring of SARS-CoV-2 in domestic animals should be sustained, especially in confirmed COVID-19 households, to identify potential infections, transmission, and host-adapted evolution.
- Public awareness about the possibility of SARS-CoV-2 transmission to domestic animals should be raised and communicated with comprehensive information to prevent public panic.
- Educating pet owners, particularly COVID-19 cases or other infectious diseases that can be transmitted between humans and animals, about proper handling of pets, and hygiene practices can help reduce the risk of transmission.
- The government or academic organizations should prepare a management protocol and animal facilities for animals infected with or at risk of next pandemic diseases to mitigate public concern.

Captive wildlife

- The standard operating protocol (SOP) on the health of staff, personnel protection equipment (PPE) practice and biosecurity measures should be

implemented and regularly validated to minimize disease introduction and transmission between staff and animals.

- The SARS-CoV-2 passive and active surveillance, as well as COVID-19 human exposure history, should be routinely conducted to provide an early warning and prevent further transmission among animal populations.
- Awareness of asymptomatic infection with potential zoonotic agents in animals should be raised and promoted in wildlife health monitoring programs, including regular health checks, clinical signs observation, and veterinary examinations that can help in the early detection and management of SARS-CoV-2 infections in animals.
- Zoo visitors and the public should be educated about animal health safety, including, appropriate physical distancing and proper hygiene practices in feeding, petting, or direct contact activities.

Free-ranging wildlife

- Active surveillance of SARS-CoV-2 and other coronaviruses should be concerned in susceptible wildlife species with close proximity to humans or in wildlife-human interface areas to determine the risk of spillover in wild animal populations.
- Passive surveillance of SARS-CoV-2 and other coronaviruses, which involves the archive of wildlife samples from animal carcasses or injured animals, should be conducted since it can emphasize the SARS-CoV-2 surveillance in free-ranging wildlife with less effort than active surveillance.
- Genetic analysis of SARS-CoV-2 isolates from wildlife species should not be neglected since it can reveal potential new important variants emerging from wildlife populations.

- Risk communications about the potential for SARS-CoV-2 introduction and its impact should be promoted. The responsible behaviors to minimize interactions, such as restricting wildlife feeding, keeping sufficient distance, and proper human waste management, should be promoted.

Further study

From the results of this study, we proposed further studies for SARS-CoV-2 and other potential zoonotic diseases, as the following:

- SARS-CoV-2 and potential zoonotic coronavirus surveillance in wildlife species in other settings for human-wildlife interface, such as in local or illegal wildlife markets and cross-border areas, should be carried out.
- SARS-CoV-2 and potential zoonotic coronavirus contamination in the environment, especially in human waste, with the possibility of transmission to rodents and macaques in Thailand, should be considered.

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