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Original Article

Predicting potential SARS-CoV-2 spillover and spillback in animals

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animal-to-human
transmissions;
Spillover and
spillback;
Infectious disease

Abstract *Background:* The COVID-19 pandemic is spreading rapidly around the world, causing countries to impose lockdowns and efforts to develop vaccines on a global scale. However, human-to-animal and animal-to-human transmission cannot be ignored, as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) can spread rapidly in farmed and wild animals. This could create a worrying cycle of SARS-CoV-2 spillover from humans to animals and spillback of new strains back into humans, rendering vaccines ineffective.

Method: This study provides a key indicator of animals that may be potential susceptible hosts for SARS-CoV-2 and coronavirus infections by analysing the phylogenetic distance between host angiotensin-converting enzyme 2 and the coronavirus spike protein. Crucially, our analysis identifies animals that are at elevated risk from a spillover and spillback incident.

Results: One group of animals has been identified as potentially susceptible to SARS-CoV-2 by harbouring a parasitic coronavirus spike protein similar to the SARS-CoV-2 spike protein. These animals may serve as amplification hosts in spillover events from zoonotic reservoirs. This group consists of a mixture of animals infected internally and naturally: minks, dogs, cats, tigers. Additionally, no internal or natural infections have been found in masked palm civet.

Conclusion: Tracing interspecies transmission in multi-host environments based solely on *in vitro* and *in vivo* examinations of animal susceptibility or serology is a time-consuming task. This approach allows rapid identification of high-risk animals to prioritize research and assessment of the risk of zoonotic disease transmission in the environment. It is a tool to rapidly identify zoonotic species that may cause outbreaks or participate in expansion cycles of coexistence with their hosts. This prevents the spread of coronavirus infections between species, preventing spillover and spillback incidents from occurring.

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Introduction

Since the outbreak of the novel coronavirus caused by the 2019 novel coronavirus (2019-nCoV)^{1,2} at the end of 2019, the virus was subsequently identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has spread rapidly throughout the country world. Since the outbreak of severe acute respiratory syndrome (SARS), it has been known that animals can become hosts for amplification by adding mutations, thereby generating new strains. For example, there was an outbreak at a mink farm that caused a mutation in SARS-CoV-2.³ Although this mutation did not lead to new strains of concern, it highlights the importance of managing spillover from human to animals to prevent spillback transfer. Currently, the susceptibility of common domestic and farmed animals to SARS-CoV-2 is established; however, there are more contacts between humans and animals than currently assessed, and a single spillover incident of an amplification host may lead to the creation of new SARS-CoV-2 strains.

In order to identify susceptible animals, the main determinants of infection must first be studied. SARS-CoV-2 shares the same cellular receptor as severe acute respiratory syndrome coronavirus (SARS-CoV), but the spike protein has a higher binding affinity^{4,5} to human angiotensin-converting enzyme 2 (hACE2), resulting in higher infectivity. Animal susceptibility has been shown to be consistent with phylogenetic grouping^{6,7}; however, most phylogenetic studies of susceptibility have been qualitative. To predict the susceptibility of animals, the phylogenetic distance of angiotensin-converting enzyme 2 (ACE2)

in each animal is determined with reference to hACE2. The distance of the coronavirus spike protein from each animal host to the first SARS-CoV-2 sequence is determined and compared together with the ACE2 distance. Our results reveal that the ACE2 of the suspected intermediate animal pangolin is more similar to humans than to bats, and the spike protein of SARS-CoV-2-related pangolin coronavirus (Pangolin-CoV) exhibits more similarity to SARS-CoV-2 than the closest bat coronavirus. Other animals have also been determined to be susceptible to SARS-CoV-2, which provides a framework for further susceptibility testing of animals to prevent spillover and spillback incidents.

Method

Sequence

ACE2 amino acid sequence is obtained from UniProt (<https://www.uniprot.org/>) and the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>). Table 1 lists the 225 ACE2 amino acid sequences.

The coronavirus spike protein amino acid sequence of the animal host and the complete coding sequence (CDS) of SARS-CoV from *Paguma larvata* (AY515512.1) are downloaded from the NCBI virus database (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/>). Only complete sequences are downloaded. Further processing is performed to remove experimental recombinant viruses. The only human coronaviruses used are SARS-CoV-2 (YP_009724390.1) and SARS-CoV (YP_009825051.1), which are used as human coronavirus references.

Table 1 List of ACE2 amino acid sequences.

Protein ID	Scientific name	Common name
Q9BYF1	<i>Homo sapiens</i>	Human
Q8R0I0, Q3URC9	<i>Mus musculus</i>	Mouse
Q5EGZ1, D3ZYK4, C7ECU5, A0A0G2JXU8	<i>Rattus norvegicus</i>	Rat
Q56NL1	<i>Paguma larvata</i>	Masked palm civet
Q5RFN1, H2PUZ5, NP_001124604.1	<i>Pongo abelii</i>	Sumatran orangutan (<i>Pongo pygmaeus abelii</i>)
Q56H28, A0A5F5XDN9	<i>Felis catus</i>	Domestic cat (<i>Felis silvestris catus</i>)
Q58DD0, A0A452DJE0, Q2HJI5	<i>Bos taurus</i>	Bovine
G1RE79	<i>Nomascus leucogenys</i>	Northern white-cheeked gibbon (<i>Hylobates leucogenys</i>)
I3M887	<i>Ictidomys tridecemlineatus</i>	Thirteen-lined ground squirrel (<i>Spermophilus tridecemlineatus</i>)
G3QWX4	<i>Gorilla gorilla gorilla</i>	Western lowland gorilla
A0A0D9RQZ0	<i>Chlorocebus sabaeus</i>	Green monkey (<i>Cercopithecus sabaeus</i>)
Q2WG88	<i>Mustela putorius furo</i>	Ferret (<i>Mustela furo</i>)
A0A2K5X283	<i>Macaca fascicularis</i>	Crab-eating macaque (Cynomolgus monkey)
J9P7Y2, F1P7C5, A0A5F4BS93, A0A5F4CXG9	<i>Canis lupus familiaris</i>	Domestic dog (<i>Canis familiaris</i>)
H0VSF6	<i>Cavia porcellus</i>	Guinea pig
G1PXH7	<i>Myotis lucifugus</i>	Little brown bat
A0A2R9BKD8, A0A2R9BJK0	<i>Pan paniscus</i>	Pygmy chimpanzee (Bonobo)

Table 1 (continued)

Protein ID	Scientific name	Common name
F6V9L3	<i>Equus caballus</i>	Horse
A0A096N4X9	<i>Papio anubis</i>	Olive baboon
W5PSB6	<i>Ovis aries</i>	Sheep
F6WXR7	<i>Monodelphis domestica</i>	Gray short-tailed opossum
G3T6Q2	<i>Loxodonta africana</i>	African bush elephant
A0A2K6NFG7	<i>Rhinopithecus roxellana</i>	Golden snub-nosed monkey (<i>Pygathrix roxellana</i>)
A0A2K5DQI6	<i>Aotus nancymae</i>	Ma's night monkey
A0A0N8EUX7	<i>Heterocephalus glaber</i>	Naked mole-rat
A0A2K6D1N8	<i>Macaca nemestrina</i>	Pig-tailed macaque
F7AH40, B6DUG6, B6DUE3, B6DUE5, B6DUF5, B6DUE6, B6DUF2, B6DUE2, B6DUG5, B6DUE4, B6DUF6, B6DUF7, B6DUE9, B6DUE8, B6DUG2, B6DUG0, B6DUF1, B6DUF9, B6DUE1, B6DUE7, B6DUF3, B6DUF8	<i>Macaca mulatta</i>	Rhesus macaque
A0A2K5ZV99	<i>Mandrillus leucophaeus</i>	Drill (<i>Papio leucophaeus</i>)
A0A2K5KSD8	<i>Cercocebus atys</i>	Sooty mangabey (<i>Cercocebus torquatus atys</i>)
A0A452EVJ5, A0A452EUV0, A0A452EVM2	<i>Capra hircus</i>	Goat
A0A1U7TY97	<i>Tarsius syrichta</i>	Philippine tarsier
A0A2Y9M9H3	<i>Delphinapterus leucas</i>	Beluga whale
K7FJ41	<i>Pelodiscus sinensis</i>	Chinese softshell turtle (<i>Trionyx sinensis</i>)
A0A2K6GHW5	<i>Propithecus coquereli</i>	Coquerel's sifaka (<i>Propithecus verreauxi coquereli</i>)
A0A452TT30, A0A384CIJ9, A0A452TT37, A0A452TTE2, A0A452TT60, A0A452TT98, A0A452TTD2, A0A452TTF7, A0A452TTE1	<i>Ursus maritimus</i>	Polar bear (<i>Thalarctos maritimus</i>)
F1NHR4, A0A5J6CU64	<i>Gallus gallus</i>	Red junglefowl
U3JP73	<i>Ficedula albicollis</i>	Collared flycatcher (<i>Muscicapa albicollis</i>)
A0A5F4W5D9, F7CNJ6	<i>Callithrix jacchus</i>	White-tufted-ear marmoset
H0WMI5	<i>Otolemur garnettii</i>	Small-eared galago (Garnett's greater bushbaby)
F7ABF9, F6PSC4, F6PSI0, F6PU11, A0A5S6MIJ1, A0A6I8QCQ6, A0A6I8SUJ0, A0A6I8S716, A0A6I8RH75	<i>Xenopus tropicalis</i>	Western clawed frog (<i>Silurana tropicalis</i>)
F7FDA2	<i>Ornithorhynchus anatinus</i>	Duckbill platypus
A0A2K6SBD4	<i>Saimiri boliviensis boliviensis</i>	Bolivian squirrel monkey
G1MC42	<i>Ailuropoda melanoleuca</i>	Giant panda
A0A1U7QTA1	<i>Mesocricetus auratus</i>	Golden hamster
G1NPB8, G5E7W8	<i>Meleagris gallopavo</i>	Wild turkey
A0A2K5PYM0	<i>Cebus capucinus imitator</i>	Panamanian white-faced capuchin
G1KTF3	<i>Anolis carolinensis</i>	Green anole (American chameleon)
A0A3Q7RAT9	<i>Vulpes vulpes</i>	Red fox
A0A2Y9S5T9	<i>Physeter macrocephalus</i>	Sperm whale (<i>Physeter catodon</i>)
A0A452R1Z9	<i>Ursus americanus</i>	American black bear (<i>Euarctos americanus</i>)
A0A1S3GHT7, A0A1S3GFD6	<i>Dipodomys ordii</i>	Ord's kangaroo rat
A0A4W2H6E0, A0A4W2H3A1	<i>Bos indicus x Bos taurus</i>	Hybrid cattle
A0A4X2M679	<i>Vombatus ursinus</i>	Common wombat

(continued on next page)

Table 1 (continued)

Protein ID	Scientific name	Common name
A0A2K6LKA0	<i>Rhinopithecus bieti</i>	Black snub-nosed monkey (<i>Pygathrix bieti</i>)
A0A2J8KU96, A0A2I3S8E3	<i>Pan troglodytes</i>	Chimpanzee
K7GLM4	<i>Sus scrofa</i>	Wild boar
A0A2K5JE65	<i>Colobus angolensis palliatus</i>	Peters' Angolan colobus
U3J4G2	<i>Anas platyrhynchos platyrhynchos</i>	Northern mallard
A0A1L8HCX9	<i>Xenopus laevis</i>	African clawed frog
H3B2W0	<i>Latimeria chalumnae</i>	Coelacanth
I3J601, A0A669DS63, A0A669B8J3, A0A669BJI9, A0A669D2Q6, A0A669F4X0	<i>Oreochromis niloticus</i>	Nile tilapia (<i>Tilapia nilotica</i>)
A0A1S3APE5	<i>Erinaceus europaeus</i>	Western European hedgehog
A0A2D0Q7Z4	<i>Ictalurus punctatus</i>	Channel catfish (<i>Silurus punctatus</i>)
A0A340Y3Y6	<i>Lipotes vexillifer</i>	Yangtze river dolphin
E7F9E5, Q5U380	<i>Danio rerio</i>	Zebrafish (<i>Brachydanio rerio</i>)
A0A2U3X0M3	<i>Odobenus rosmarus divergens</i>	Pacific walrus
A0A3Q0H852, A0A3Q0H3J6	<i>Alligator sinensis</i>	Chinese alligator
A0A452CBT6	<i>Balaenoptera acutorostrata scammoni</i>	North Pacific minke whale (<i>Balaenoptera davidsoni</i>)
A0A3Q7TE16	<i>Ursus arctos horribilis</i>	Grizzly bear
Q1LZX8	<i>Chlorocebus aethiops</i>	Green monkey (<i>Cercopithecus aethiops</i>)
E2DHI3, ADN93471.1	<i>Rhinolophus macrotis</i>	Big-eared horseshoe bat
A4PIG8, D8WU01	<i>Rousettus leschenaultii</i>	Leschenault's rousette
Q2PGE1	<i>Procyon lotor</i>	Raccoon
E2DHI4, E2DHI7, U5WHY8	<i>Rhinolophus sinicus</i>	Chinese rufous horseshoe bat
E2DHI9	<i>Rhinolophus pusillus</i>	Least horseshoe bat
E2DHI2, B6ZGN7, A0A671F9Q9, A0A671F0T6	<i>Rhinolophus ferrumequinum</i>	Greater horseshoe bat
B4XEP4	<i>Nyctereutes procyonoides</i>	Raccoon dog (<i>Canis procyonoides</i>)
A0A3Q7N3M7	<i>Callorhinus ursinus</i>	Northern fur seal
A0A341BCI8	<i>Neophocaena asiaeorientalis asiaeorientalis</i>	Yangtze finless porpoise (<i>Neophocaena phocaenoides asiaeorientalis</i>)
A0A2Y9GBR2, A0A2Y9GEI9	<i>Neomonachus schauinslandi</i>	Hawaiian monk seal (<i>Monachus schauinslandi</i>)
A0A4W4EE33, A0A4W4EFY7	<i>Electrophorus electricus</i>	Electric eel (<i>Gymnotus electricus</i>)
A0A663EPL4	<i>Aquila chrysaetos chrysaetos</i>	Golden eagle
A0A5P9VP25	<i>Tadorna cana</i>	South African shelduck
A0A667IF49	<i>Lynx canadensis</i>	Canada lynx
A0A220QT48	<i>Sus scrofa domesticus</i>	Domestic pig
A0A673UPR4	<i>Suricata suricatta</i>	Meerkat
A0A663M979, A0A663M8Y2, A0A663M7K6, A0A663M8A8	<i>Athene cunicularia</i>	Burrowing owl (<i>Speotyto cunicularia</i>)
A0A6C0PIH2	<i>Oryctolagus cuniculus</i>	European rabbit
A0A151N089	<i>Alligator mississippiensis</i>	American alligator
A0A1A8AXC5	<i>Nothobranchius furzeri</i>	Turquoise killifish
H0ZCK6, H0ZYW8, A0A674GKE4, A0A674GHV0, A0A674GDZ7, A0A674GJP6	<i>Taeniopygia guttata</i>	Zebra finch (<i>Poephila guttata</i>)
A0A1V4JC49	<i>Patagioenas fasciata monilis</i>	Band-tailed pigeon
A0A665VWQ8, A0A665VWR3	<i>Echeneis naucrates</i>	Live sharksucker
A0A4Z2GEX4	<i>Liparis tanakae</i>	Tanaka's snailfish
A0A669PPG5, A0A669PSZ2, A0A669Q5K7, A0A669PKV0, A0A669Q5M4	<i>Phasianus colchicus</i>	Common pheasant
A0A671T498	<i>Sinocyclocheilus anshuiensis</i>	Anshui golden thread catfish

Table 1 (continued)

Protein ID	Scientific name	Common name
A0A4W3HYJ6, A0A4W3I547, A0A4W3I1M1, A0A4W3HYM0, A0A4W3I1M6, A0A4W3IPJ3, A0A4W3HYL1, A0A4W3IPI8, A0A4W3HYL6	<i>Callorhinchus milii</i>	Ghost shark
A0A667X0J3	<i>Myripristis murdjan</i>	Pinecone soldierfish
A0A218UNR1	<i>Lonchura striata domestica</i>	Bengalese finch
A0A2I0MLI2	<i>Columba livia</i>	Rock dove
A0A672V5V3	<i>Strigops habroptila</i>	Kakapo
A0A1S3SF35	<i>Salmo salar</i>	Atlantic salmon
A0A2I4D9L3	<i>Austrofundulus limnaeus</i>	Limnaeus Killifish
V8NIH2	<i>Ophiophagus hannah</i>	King cobra (<i>Naja hannah</i>)
A0A0S7FTS4	<i>Poeciliopsis prolifica</i>	Blackstripe livebearer
A0A670YAG2	<i>Pseudonaja textilis</i>	Eastern brown snake
A0A2U4AJL3	<i>Tursiops truncatus</i>	Atlantic bottle-nosed dolphin (<i>Delphinus truncatus</i>)
A0A0K8U7D5	<i>Bactrocera latifrons</i>	Malaysian fruit fly (<i>Chaetodacus latifrons</i>)
A0A2S2POH8	<i>Schizaphis graminum</i>	Greenbug aphid
A0A2H8TEU2	<i>Melanaphis sacchari</i>	Sugarcane aphid
QLF98521.1	<i>Melogale moschata</i>	Chinese ferret-badger
QLF98526.1	<i>Arctonyx collaris</i>	Hog badger
QLF98520.1	<i>Tadarida brasiliensis</i>	Mexican free-tailed bat
ACT66266.1	<i>Pipistrellus abramus</i>	Japanese house bat
XP_017505752.1	<i>Manis javanica</i>	Pangolin
QLH93383.1	<i>Manis pentadactyla</i>	Chinese pangolin
QNC68911.1	<i>Mustela lutreola biedermanni</i>	European mink
ACT66274.1	<i>Phodopus campbelli</i>	Campbell's dwarf hamster
ACT66267.1	<i>Chinchilla lanigera</i>	Long-tailed chinchilla
QKE49997.1	<i>Cynopterus sphinx</i>	Greater short-nosed fruit bat
QKE49998.1	<i>Megaderma lyra</i>	Greater false vampire bat
QNV47311.1	<i>Tupaia glis</i>	Common tree shrew
XP_006164754.1	<i>Tupaia belangeri chinensis</i>	Chinese tree shrew
XP_032187679.1	<i>Mustela erminea</i>	Short-tailed weasel
XP_031301717.1	<i>Camelus dromedarius</i>	Arabian camel
ABU54053.1	<i>Rhinolophus pearsonii</i>	Pearson's horseshoe bat
XP_011361275.1	<i>Pteropus vampyrus</i>	Large flying fox
XP_020768965.1	<i>Odocoileus virginianus</i>	White-tailed deer
XP_007090142.2	<i>Panthera tigris</i>	Tiger

Alignment and phylogenetic tree generation

Since the complete CDS of SARS-CoV from *P. larvata* (AY515512.1) does not contain an annotation for the spike protein, this sequence is aligned with the SARS Tor2 spike protein (NC_004718.3:21492-25259) to obtain the spike protein sequence. Sequences are translated into amino acid sequences using MUSCLE.⁸ ACE2 alignment^{9–13} is performed by Nextflow¹⁴ using MAFFT,¹⁵ and phylogenetic trees are generated using IQ-TREE¹⁶ with ultrafast bootstrap¹⁷ and ModelFinder Plus.¹⁸ Both ACE2 and spike protein phylogenetic trees are generated using JTT+F+R6 and WAG+F+R10 models of ACE2 and spike protein sequences, respectively, and selected according to the Bayesian information criterion of ModelFinder Plus.

Obtain phylogenetic distance

Phylogenetic distances are obtained from tree files using the Python package TreeSwift.¹⁹ All ACE2 distances are measured from node to hACE2 node (UniProtKB-Q9BYF1). Zoonotic coronavirus spike protein distances are measured from node SARS-CoV-2 (YP_009724390.1) and SARS-CoV (YP_009825051.1) spike proteins. For ACE2 sequences, intraspecific phylogenetic distances are determined to be close (Fig. 1); therefore, each animal is represented as an average.

In order to compare the phylogenetic distance between ACE2 and spike protein, each animal must have its own ACE2 and parasitic coronavirus spike protein sequences (Table 2). Several subspecies are chosen as representatives of the species. From the coronavirus spike protein

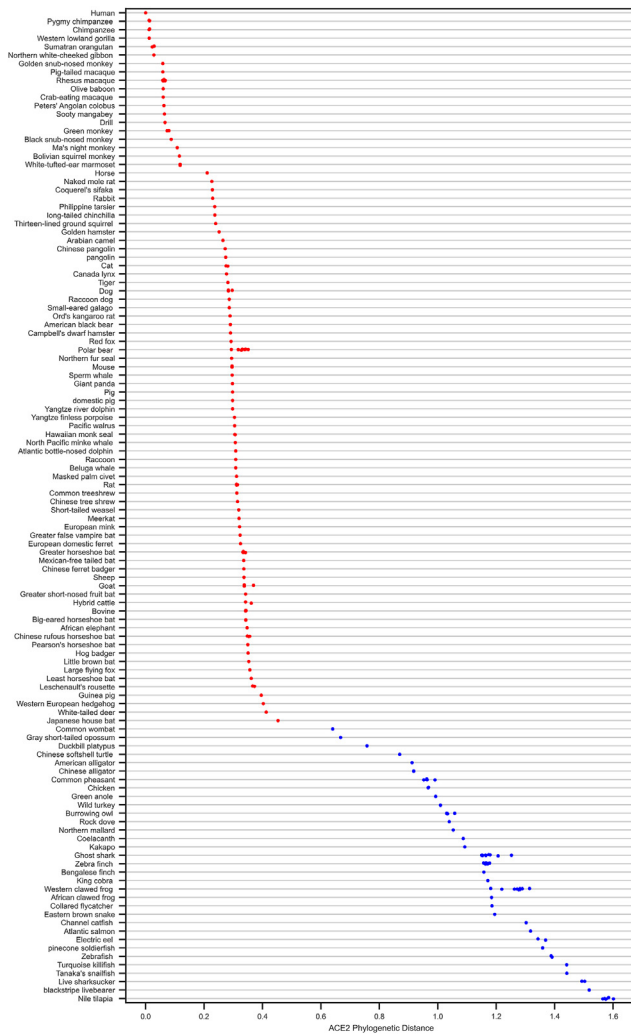


Fig. 1. Distribution of phylogenetic distance of animal ACE2 relative to hACE2. Only animals with multiple ACE2 sequences are shown and grouped according to phylogenetic distance from hACE2: close to humans (red <0.5), distant from humans ($0.5 \leq$ blue ≤ 2). ACE2 is extremely narrowly distributed in each animal.

sequence, *Pan troglodytes verus* stands for *P. troglodytes*. From the ACE2 sequence, *Mustela lutreola biedermanni* represents *M. lutreola*, and *Lonchura striata domestica* represents *L. striata*.

Results

Animal susceptibility

To investigate the correlation between animal susceptibility and ACE2 similarity, a phylogenetic analysis of human and animal ACE2 sequences is performed. A total of 225 sequences (130 unique species) obtained from UniProt and NCBI databases are included in the analysis. From the phylogenetic tree file, the similarity of each sequence to hACE2 is calculated by the distance of each branch between human and animal nodes. Comparing known animal

susceptibility to ACE2 phylogenetic distances reveals that all SARS-CoV-2 susceptible animals known to date^{20–27} have ACE2 distances below 0.41 (Table 3). The non-susceptible *Gallus gallus* (red junglefowl) has an ACE2 distance of 0.94 and is significantly further away from humans and susceptible animals. The only exception is *Sus scrofa domestica* (domestic pig), whose ACE2 distance is within the range of all susceptible animals, but no viral shedding or ribonucleic acid (RNA) is detected. The ACE2 distances observed in animals can be divided into three groups: animals with an ACE2 distance within 0.5 in humans, animals with an ACE2 distance above 0.5 and below 2, and finally animals with an ACE2 distance above 2 (Fig. 2). Our analysis of the ACE2 phylogenetic distance with reference to hACE2 reveals that the susceptible animals identified to date belong to the first group closest to hACE2.

Predicting animals at risk of human spillover

To identify potential animals that may serve as amplification hosts for SARS-CoV-2, the role of phylogenetic distance of the coronavirus spike protein in cross-species jumping is further investigated by analysing together with the ACE2 distance (Fig. 3). Phylogenetic analysis of the complete sequence is studied to trace the evolutionary history of SARS-CoV-2^{28,29}; however, in order to understand short-term cross-species transmission rather than long-term evolutionary history,³⁰ only the spike protein is analysed as it is the main determinant of susceptibility. The 2105 complete spike protein sequences of coronaviruses used in this analysis are obtained from the NCBI virus database.³¹ ACE2 in each animal is associated with the spike protein of the coronavirus found in that particular animal, which is known as a parasitic coronavirus.

The SARS-CoV-2 reference sequence Wuhan-Hu-1 (NC_045512.2) and spike protein (YP_009724390.1) were used as a reference to identify potential intermediate hosts or amplification hosts of the COVID-19 pandemic (Fig. 3). Pangolins are suspected to be the intermediate host,^{32,33} and their ACE2 shares similarities with *Rhinolophus* bats and hACE2. As with the SARS-CoV transmission pathway, this jump increases the similarity between the ACE2 and spike protein sequences compared to hACE2 and the first SARS-CoV-2 spike protein sequence. A closer similarity to Pangolin-CoV suggests that SARS-CoV-2 adopts the spike protein of Pangolin-CoV, allowing recombination to occur. Animals confirmed to be susceptible (Table 3) have an ACE2 distance of 0.41 and below. The ACE2 distance of these susceptible animals belongs to the first group (ACE2 distance <0.5). This is consistent with the intermediate animals of SARS-CoV and suspected SARS-CoV-2, namely masked palm civets and pangolins, whose ACE2 distances are 0.30 and 0.27, respectively. Thus, it is speculated that animals with an ACE2 distance less than 0.5 may be susceptible to SARS-CoV-2 infection (Table 4). Animals that have been identified as susceptible (Fig. 3b) are at elevated risk of playing a role in a spillover incident. In addition to this, animals that have not been identified as susceptible (Fig. 3c) should not be ignored. Since their ACE2 is closer to humans, they also at risk of infection. The nature of this analysis depends on the range of zoonotic coronaviruses

Table 2 List of coronavirus spike protein sequences.

Protein ID	Host	Common name	Species
AWW13519.1	<i>Pan troglodytes</i>	Chimpanzee	<i>Betacoronavirus 1</i>
ADC35511.1	<i>Chlorocebus aethiops</i>	Green monkey (<i>Cercopithecus aethiops</i>)	Severe acute respiratory syndrome–related coronavirus (SARSr-CoV)
BAS18866.1	<i>Equus caballus</i>	Horse	<i>Betacoronavirus 1</i>
AFE48795.1	<i>Oryctolagus cuniculus</i>	Rabbit	Rabbit coronavirus HKU14
QMT97936.1	<i>Mesocricetus auratus</i>	Golden hamster	SARSr-CoV
AVN89334.1	<i>Camelus dromedarius</i>	Arabian camel	Middle East respiratory syndrome–related coronavirus
QJA48614.1	<i>Manis javanica</i>	Pangolin	Pangolin-CoV
QLG96797.1	<i>Felis catus</i>	Domestic cat (<i>Felis silvestris catus</i>)	SARSr-CoV
QLC48491.1	<i>Panthera tigris</i>	Tiger	SARSr-CoV
QJT08292.1	<i>Canis lupus familiaris</i>	Domestic dog (<i>Canis familiaris</i>)	SARSr-CoV
ACN89742.1	<i>Mus musculus</i>	Mouse	Murine coronavirus
AUF40275.1	<i>Sus scrofa</i>	Wild boar	<i>Betacoronavirus 1</i>
BAT33329.1	<i>Sus scrofa domesticus</i>	Domestic pig	Porcine epidemic diarrhea virus
QII89061.1	<i>Tursiops truncatus</i>	Atlantic bottle-nosed dolphin (<i>Delphinus truncatus</i>)	Bottlenose dolphin coronavirus
ABW87820.1	<i>Delphinapterus leucas</i>	Beluga whale	Beluga whale coronavirus SW1
AY515512.1	<i>Paguma larvata</i>	Masked palm civet	SARSr-CoV
AJA91207.1	<i>Rattus norvegicus</i>	Rat	China Rattus coronavirus HKU24
QJS39579.1	<i>Mustela lutreola biedermanni</i>	European mink	SARSr-CoV
ASR18938.1	<i>Mustela putorius furo</i>	Ferret (<i>Mustela furo</i>)	Ferret coronavirus
ATO98145.1	<i>Rhinolophus ferrumequinum</i>	Greater horseshoe bat	SARSr-CoV
QDF43810.1	<i>Cynopterus sphinx</i>	Greater short-nosed fruit bat	Coronavirus BtRs-AlphaCoV/YN2018
ACT10983.1	<i>Bos taurus</i>	Bovine	<i>Betacoronavirus 1</i>
ABD75332.1	<i>Rhinolophus macrotis</i>	Big-eared horseshoe bat	SARSr-CoV
ATO98205.1	<i>Rhinolophus sinicus</i>	Chinese rufous horseshoe bat	SARSr-CoV
ASL24654.1	<i>Myotis lucifugus</i>	Little brown bat	Bat coronavirus CDPHE15
AVP78031.1	<i>Rhinolophus pusillus</i>	Least horseshoe bat	SARSr-CoV
AOG30822.1	<i>Rousettus leschenaultii</i>	Leschenault's rousette	Rousettus bat coronavirus GCCDC1
AGX27810.1	<i>Erinaceus europaeus</i>	Western European hedgehog	Hedgehog coronavirus 1
ACJ66977.1	<i>Odocoileus virginianus</i>	White-tailed deer	<i>Betacoronavirus 1</i>
AIA62343.1	<i>Pipistrellus abramus</i>	Japanese house bat	Pipistrellus bat coronavirus HKU5
ASM61973.1	<i>Gallus gallus</i>	Red junglefowl	Avian coronavirus
ACV87276.1	<i>Meleagris gallopavo</i>	Wild turkey	Avian coronavirus

that have been sequenced and made available in the database. Many animals belonging to this group may carry coronaviruses similar to SARS-CoV-2 but have not yet been identified.

In April 2020, SARS-CoV-2 infection was detected in farmed mink in the Netherlands.^{3,34} Mink-related mutations found in humans confirm spillover incidents from humans to mink.^{35,36} In samples from feral cats near ten farms, antibodies and viral RNA were detected in the cats.^{3,34} As shown in Fig. 4, SARS-CoV-2 has the potential to be transmitted into farmed minks through workers (Fig. 4a). Due to the close proximity of farmed minks and wandering stray cats, these animals were co-infected (Fig. 4b). When workers came into contact with farmed minks, the mutated variants were spilled back into the human cycle. In summary, our analysis of the phylogenetic distance between ACE2 and the parasitic coronavirus spike protein provides a key indicator of animals that may be susceptible to SARS-

CoV-2 infection and, most importantly, those animals at elevated risk of spillover and spillback.

Discussion

The cellular receptor ACE2 is critical for SARS-CoV-2 binding to cells, but only after the spike protein is cleaved by the transmembrane protease, serine 2 (TMPRSS2) and furin.^{37–39} Hence, the location and abundance of ACE2 expression determines the tropism for SARS-CoV-2 infection, as well as the presence of TMPRSS2 and furin, which are major determinants of whether an animal is susceptible to infection. A study comparing specific binding residues in hACE2 showed that pig ACE2 differs from humans at specific sites, explaining why pigs are resistant to infection.⁴⁰ Nevertheless, all animals confirmed to be susceptible to date exhibit a similar characteristic; namely, ACE2 phylogenetic distance below 0.5.

Table 3 ACE2 phylogenetic distance is lower in susceptible animals. To date, most animals known to be susceptible have been experimentally infected. Naturally infected animals include tiger, European mink, and white-tailed deer. Susceptible animals have an ACE2 distance of 0.41 or less.

Animal	Common name	Clinical presentation	Susceptibility	Phylogenetic distance
<i>Macaca mulatta</i>	Rhesus macaque	5 days post-infection (dpi), mild to moderate symptoms. ²⁴	Yes	0.06
<i>Oryctolagus cuniculus</i>	European rabbit	10 ⁶ fifty-percent tissue culture infective doses (TCID ₅₀). No clinical symptoms. Viral RNA peak 2 dpi nose/throat. ²²	Yes	0.22
<i>Mesocricetus auratus</i>	Golden hamster	10 ⁵ plaque-forming units (PFU), 100 µL. Viral RNA detected at 2 dpi. Transmission presents. ²⁵	Yes	0.25
<i>Felis catus</i>	Domestic cat	10 ⁵ PFU. Viral RNA detected in subadult cat feces at 3 dpi. Viral RNA detected in juvenile cat nasal wash at 2 dpi. ²⁰	Yes	0.27
<i>Panthera tigris</i>	Tiger	Natural infection. ²⁷	Yes	0.28
<i>Canis lupus familiaris</i>	Domestic dog	10 ⁵ PFU. Viral RNA detected in feces at 2 dpi. Negative nasal swab. ²⁰	Yes	0.28
<i>Sus scrofa domesticus</i>	Domestic pig	Not susceptible ²⁰	No	0.29
<i>Tupaia belangeri chinensis</i>	Chinese treeshrew	10 ⁶ PFU, 1 mL. Viral RNA detected at 6 dpi (first sample), shedding. ²³	Yes	0.31
<i>Mustela lutreola biedermani</i>	European mink	Natural infection. ³	Yes	0.31
<i>Mustela putorius furo</i>	Ferret	1. 10 ⁵ TCID ₅₀ . Shedding detected in nasal wash at 2 dpi. Peak viral RNA nasal wash at 2 dpi. ²⁰ 2. 10 ⁵ PFU. Peak viral RNA nasal wash at 4 dpi, limited replication in other organs. ²¹	Yes	0.32
<i>Odocoileus virginianus</i>	White-tailed deer	Natural infection. ²⁶	Yes	0.41
<i>Gallus gallus</i>	Red junglefowl	Not susceptible ^{20,21}	No	0.94

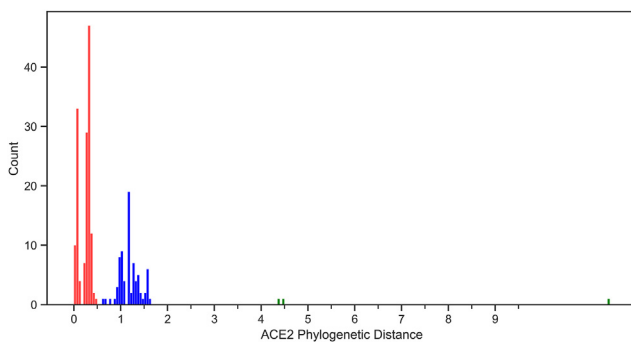


Fig. 2. Distribution of animal ACE2 phylogenetic distances. In most animals, the branch distance between ACE2 and hACE2 is less than 1.5. Three distinct groups of distribution can be observed: animal ACE2 close to humans (red <math>< 0.5</math>), distant from humans (Melanaphis sacchari (sugarcane aphid), *Schizaphis graminum* (greenbug aphid), and *Bactrocera latifrons* (Malaysian fruit fly) (*Chaetodacus latifrons*).

Two components are provided to determine the potential of an animal to serve as an amplification host: the distance between ACE2 and hACE2 as well as the distance between the parasitic protein and the SARS-CoV-2 spike protein. The path of transmission to humans starts with the bat, which is the natural reservoir, and then jumps downward to an intermediate animal that is to the left of both SARS-CoV and SARS-CoV-2. This suggests that potential intermediate animals have two characteristics. First, its ACE2 is more similar to hACE2, thereby reducing the cross-species barrier. Second, the parasitic coronavirus spike protein shares more similarities with SARS-CoV-2. This enables the platform for recombination to occur, which, coupled with the smaller ACE2 gap, could lead to the creation of a new type of coronavirus. To date, there are two known large-scale human-to-animal spillover events: the mink farm in the Netherlands and the white-tailed deer in the United States. Both animals are in close contact with humans; however, only the mink farm incident resulted in a spillback event of SARS-CoV-2 back to 68 % of farm workers,³⁵ whereas white-tailed deer had 3 % (3/92) event of spillback to humans.²⁶ Judging from the results in Fig. 3,

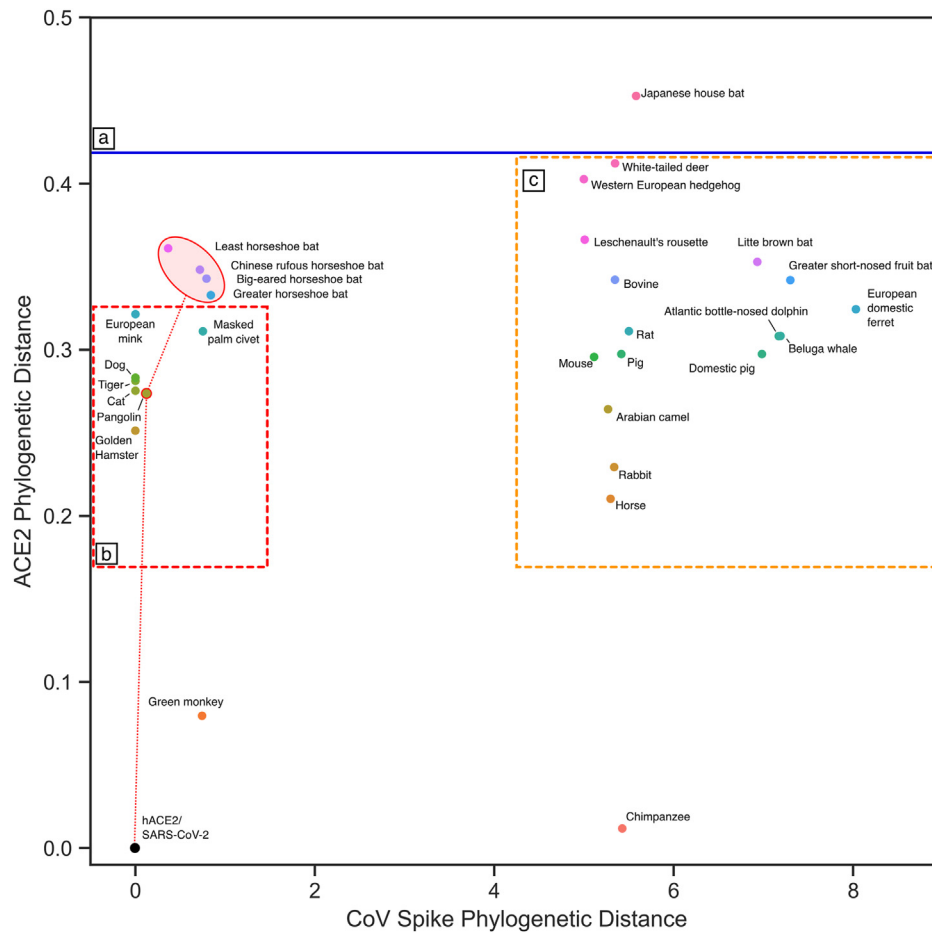


Fig. 3. Phylogenetic distance between animal ACE2 and the respective parasitic coronavirus spike protein with reference to hACE2 and SARS-CoV-2. Only animals with ACE2 below 0.5 (group closest to hACE2) are shown. Potential amplification hosts of SARS-CoV-2: a, animals below the susceptibility line have closer ACE2 similarity to humans. This zone is categorised into two areas: b, elevated risk of SARS-CoV-2 mutation amplification (red box) and c, potential risk (orange box). High-risk animals are susceptible based on the ACE2 similarity and have a parasitic coronavirus spike protein similar to SARS-CoV-2. ACE2, which belongs to animals with unconfirmed potential, is to humans; however, there is no parasitic coronavirus spike protein similar to SARS-CoV-2.

Table 4 Animals may be susceptible to SARS-CoV-2 based on an ACE2 distance between 0.5 and humans.

Scientific Name	Common Name	ACE2 Distance
<i>Pan paniscus</i>	Pygmy chimpanzee	0.012
<i>Pan troglodytes</i>	Chimpanzee	0.012
<i>Gorilla gorilla gorilla</i>	Western lowland gorilla	0.012
<i>Pongo abelii</i>	Sumatran orangutan	0.023
<i>Nomascus leucogenys</i>	Northern white-cheeked gibbon	0.028
<i>Rhinopithecus roxellana</i>	Golden snub-nosed monkey	0.059
<i>Macaca mulatta</i>	Rhesus macaque	0.059
<i>Macaca nemestrina</i>	Pig-tailed macaque	0.059
<i>Papio anubis</i>	Olive baboon	0.060
<i>Macaca fascicularis</i>	Crab-eating macaque	0.061
<i>Colobus angolensis palliatus</i>	Peters' Angolan colobus	0.063
<i>Cercocebus atys</i>	Sooty mangabey	0.065
<i>Mandrillus leucophaeus</i>	Drill	0.066
<i>Chlorocebus sabaeus</i>	Green monkey	0.074
<i>Rhinopithecus bieti</i>	Black snub-nosed monkey	0.090

(continued on next page)

Table 4 (continued)

Scientific Name	Common Name	ACE2 Distance
<i>Cebus capucinus imitator</i>	Panamanian white-faced capuchin	0.101
<i>Aotus nancymae</i>	Ma's night monkey	0.109
<i>Saimiri boliviensis boliviensis</i>	Bolivian squirrel monkey	0.117
<i>Callithrix jacchus</i>	White-tufted-ear marmoset	0.119
<i>Equus caballus</i>	Horse	0.213
<i>Heterocephalus glaber</i>	Naked mole-rat	0.229
<i>Propithecus coquereli</i>	Coquerel's sifaka	0.231
<i>Oryctolagus cuniculus</i>	European rabbit	0.232
<i>Chinchilla lanigera</i>	Long-tailed chinchilla	0.238
<i>Tarsius syrichta</i>	Philippine tarsier	0.238
<i>Ictidomys tridecemlineatus</i>	Thirteen-lined ground squirrel	0.242
<i>Mesocricetus auratus</i>	Golden hamster	0.254
<i>Camelus dromedarius</i>	Arabian camel	0.267
<i>Manis pentadactyla</i>	Chinese pangolin	0.274
<i>Manis javanica</i>	Pangolin	0.276
<i>Felis catus</i>	Domestic cat	0.277
<i>Lynx canadensis</i>	Canada lynx	0.278
<i>Panthera tigris</i>	Tiger	0.283
<i>Canis lupus familiaris</i>	Domestic dog	0.285
<i>Nyctereutes procyonoides</i>	Raccoon dog	0.288
<i>Otolemur garnettii</i>	Small-eared galago	0.290
<i>Ursus americanus</i>	American black bear	0.292
<i>Dipodomys ordii</i>	Ord's kangaroo rat	0.292
<i>Phodopus campbelli</i>	Campbell's dwarf hamster	0.293
<i>Ursus arctos horribilis</i>	Grizzly bear	0.293
<i>Vulpes vulpes</i>	Red fox	0.294
<i>Ursus maritimus</i>	Polar bear	0.296
<i>Callorhinus ursinus</i>	Northern fur seal	0.296
<i>Mus musculus</i>	Mouse	0.298
<i>Physeter macrocephalus</i>	Sperm whale	0.299
<i>Lipotes vexillifer</i>	Yangtze river dolphin	0.300
<i>Ailuropoda melanoleuca</i>	Giant panda	0.301
<i>Odobenus rosmarus divergens</i>	Pacific walrus	0.306
<i>Neophocaena asiaorientalis</i>	Yangtze finless porpoise	0.306
<i>Neomonachus schauinslandi</i>	Hawaiian monk seal	0.307
<i>Balaenoptera acutorostrata scammoni</i>	North Pacific minke whale	0.310
<i>Procyon lotor</i>	Raccoon	0.310
<i>Tursiops truncatus</i>	Atlantic bottle-nosed dolphin	0.311
<i>Delphinapterus leucas</i>	Beluga whale	0.311
<i>Paguma larvata</i>	Masked palm civet	0.313
<i>Rattus norvegicus</i>	Rat	0.314
<i>Tupaia glis</i>	Common treeshrew	0.315
<i>Tupaia belangeri chinensis</i>	Chinese treeshrew	0.318
<i>Mustela erminea</i>	Short-tailed weasel	0.321
<i>Suricata suricatta</i>	Meerkat	0.321
<i>Mustela lutreola biedermanni</i>	European mink	0.324
<i>Megaderma lyra</i>	Greater false vampire bat	0.325
<i>Mustela putorius furo</i>	Ferret	0.327
<i>Rhinolophus ferrumequinum</i>	Greater horseshoe bat	0.336
<i>Tadarida brasiliensis</i>	Mexican free-tailed bat	0.338
<i>Melogale moschata</i>	Chinese ferret-badger	0.338
<i>Ovis aries</i>	Sheep	0.339
<i>Capra hircus</i>	Goat	0.340
<i>Bos indicus x Bos taurus</i>	Hybrid cattle	0.344
<i>Bos taurus</i>	Bovine	0.345
<i>Rhinolophus macrotis</i>	Big-eared horseshoe bat	0.346
<i>Pteropus vampyrus</i>	Large flying fox	0.346
<i>Loxodonta africana</i>	African bush elephant	0.349

Table 4 (continued)

Scientific Name	Common Name	ACE2 Distance
<i>Rhinolophus sinicus</i>	Chinese rufous horseshoe bat	0.352
<i>Rhinolophus pearsonii</i>	Pearson's horseshoe bat	0.353
<i>Arctonyx collaris</i>	Hog badger	0.353
<i>Cynopterus sphinx</i>	Greater short-nosed fruit bat	0.355
<i>Myotis lucifugus</i>	Little brown bat	0.357
<i>Rhinolophus pusillus</i>	Least horseshoe bat	0.365
<i>Rousettus leschenaultii</i>	Leschenault's rousette	0.370
<i>Cavia porcellus</i>	Guinea pig	0.393
<i>Erinaceus europaeus</i>	Western European hedgehog	0.407
<i>Odocoileus virginianus</i>	White-tailed deer	0.408
<i>Pipistrellus abramus</i>	Japanese house bat	0.457

European mink is closer to the parasitic coronavirus spike distance of SARS-CoV-2 than white-tailed deer. This suggests that animals in close proximity to ACE2 and the parasitic coronavirus spike distance have a higher risk of spillover from humans and spillback to humans and can readily serve as amplification hosts.

Besides the intermediate host, there are multiple animals with the same characteristics: ACE2, which is similar to humans, and the parasitic coronavirus spike protein, which is similar to SARS-CoV-2. This raises a similar question to the previous SARS outbreak: Are there multiple intermediate hosts?^{41,42} Two other animals, the raccoon dog (*Nyctereutes procyonoides*) and the Chinese ferret-badger

(*Melogale moschata*), have also been found to be infected with SARS-CoV.⁴¹ These findings encourage further studies to determine whether multiple animals played a role in the outbreak of the novel coronavirus in 2019. Nevertheless, regardless of whether they played a role in the outbreak of the novel coronavirus in 2019, these animals could serve as amplification hosts in a spillover incident. As shown in Fig. 4, these animals have been identified as susceptible and can be used as a guide for monitoring potential spillover incidents, particularly for domestic farmed animals. The recent mink spillover and spillback incident did not raise new strain of concern; however, further spillover and spillback incidents could lead to the emergence of new SARS-CoV-2 strains. Such an oversight could render SARS-CoV-2 vaccines ineffective.

Conclusion

Our *in-silico* analysis is not a substitute for *in vitro* and *in vivo* studies of animal susceptibility or serology to confirm intermediate animals prior to an outbreak; however, it is a rapid way to identify zoonotic species that may be responsible for an outbreak or involved in spillover and spillback events. Since animal acquisition and testing is a daunting task, this approach can rapidly identify animals at high-risk to prioritise research and assess the risk of zoonotic amplification in the environment. These data were obtained from coronaviruses discovered in zoonotic hosts and in the ACE2 databases prior to the SARS-CoV-2 outbreak, except for the first reference to the Wuhan SARS-CoV-2 spike sequence. Without the aid of experimental studies, the method identifies a variety of animals that are subsequently shown to be susceptible to SARS-CoV-2 infection. This highlights the advantages of leveraging the vast amount of sequence data in the databases as a rapid response tool in the initial stages of a pandemic.

Availability of data and material

All data are available from the NCBI virus database. Next-flow code is accessible on GitHub (upon confirmation of release).

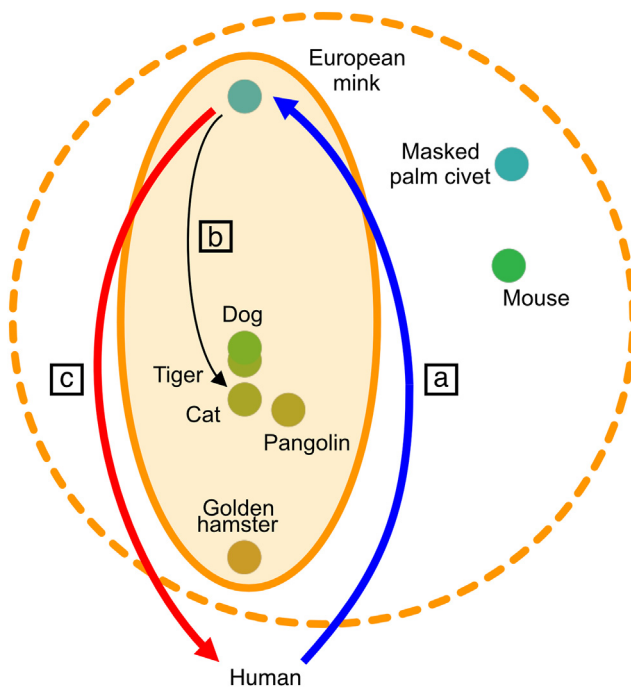


Fig. 4. Amplification cycle of minks and cats in Netherlands. a, Spillover incident of farmed minks or stray cats. b, Farmed minks and stray cats are co-infected due to being too close together. c, The spillback incident occurred when farm workers were managing minks nearby.

Abbreviations

SARS-CoV-2 Severe acute respiratory syndrome coronavirus 2
 SARS Severe acute respiratory syndrome
 SARS-CoV Severe acute respiratory syndrome coronavirus
 hACE2 Human angiotensin-converting enzyme 2
 ACE2 Angiotensin-converting enzyme 2
 Pangolin-CoV SARS-CoV-2-related pangolin coronavirus
 NCBI National Center for Biotechnology Information
 CDS Coding sequence
 SARSr-CoV Severe acute respiratory syndrome-related coronavirus
 RNA Ribonucleic acid
 dpi Days post-infection
 TCID Tissue culture infective doses
 PFU Plaque-forming units
 TMPRSS2 Transmembrane protease, serine 2

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