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RESEARCH ARTICLE

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Comparative analyses of ACE2 and TMPRSS2 gene: Implications for the risk to which vertebrate animals are susceptible to SARS-CoV-2

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Abstract

Along with the control and prevention of coronavirus disease 2019 transmission, infected animals might have potential to carry the virus to spark new outbreaks. However, very few studies explore the susceptibility of animals to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Viral attachment as a crucial step for cross-species infection requires angiotensin-converting enzyme 2 (ACE2) as a receptor and depends on TMPRSS2 protease activity. Here, we searched the genomes of metazoans from different classes using an extensive BLASTP survey and found ACE2 and TMPRSS2 occur in vertebrates, but some vertebrates lack Tmprss2. We identified 6 amino acids among 25 known human ACE2 residues are highly associated with the binding of ACE2 to SARS-CoV-2 (p value < .01) by Fisher exact test, and following this, calculated the probability of viral attachment within each species by the randomForest function from R randomForest library. Furthermore, we observed that Ace2 selected from seven animals based on the above analysis lack the hydrophobic contacts identified on human ACE2, indicating less affinity of SARS-CoV-2 to Ace2 in animals than humans. Finally, the alignment of 3D structure between human ACE2 and other animals by I-TASSER and TM-align displayed a reasonable structure for viral attachment within these species. Taken together, our data may shed light on the human-to-animal transmission of SARS-CoV-2.

KEYWORDS
ACE2, infection, livestock, SARS-CoV-2, TMPRSS2

1 | INTRODUCTION

An unusual pneumonia caused by human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus with ~79% similarity of the genome sequence with SARS-CoV, is threatening global public health and economy.[1–3] This disease was named coronavirus disease 2019 (COVID-19) by the World Health Organization. Until March 9, 2021, SARS-CoV-2 has spread to more than 188 countries and regions and caused 117,236,336 COVID-19 positive cases with 2,603,942 deaths (https://coronavirus.jhu.edu/map.html).

The origin and transmission routes of SARS-CoV-2 is still unclear. Generally, other coronaviruses SARS-CoV and Middle East respiratory syndrome coronavirus transmitted from bats to an intermediate host and then spillover to humans.[4–6] Bats are considered as the original host of SARS-CoV-2 according to isolation of bat coronavirus RaTG13, which has 96.1% genome identity with SARS-CoV-2.[7,8] Malayan pangolin is a potential intermediate host of SARS-CoV-2 because of the isolation of pangolin-CoV with 100%, 98.6%, 97.8%, and 90.7% amino acid identity with human SARS-CoV-2 in the E, M, N, and S proteins. Meanwhile, infected pangolins showed

clinical signs and circulating antibodies against pangolin-CoV, which can react with S protein of SARS-CoV-2.[9–11]

In addition to the unclear molecular events in which intermediate host during bat-to-human transmission of SARS-CoV-2, the transmission of SARS-Cov-2 between human and other mammals is becoming the new concern along with more and more reported emerging of cases in animals. In the experimental approach, rhesus macaques, golden hamster, domestic cats, dogs, ferret, Egyptian fruit bat, and rabbit are susceptible to infection by SARS-CoV-2.[12–28] To date, pet dogs and cats in contact with COVID-19 patients,[29] lion and tiger in New York city zoo,[30] and mink on farms in the Netherlands have tested positive for COVID-19.[31–33] At this moment, the risk of catching SARS-CoV-2 from an infected animal is much lower than from COVID-19 positive person. Nevertheless, along with the restrictions on the movement of COVID-19 person and numbers of infected people fall, the virus might jump back and forth between humans and animals to spark a new outbreak. Therefore, to effectively prevent new spillovers to control future pandemics, more studies should be carried out to understand the susceptibility of the virus to various livestock.

Effective SARS-CoV-2 cell entry is the first line for viral infection and determines the host range. Coronaviruses entry into target cells depends on: (1) the binding of the spike (S) protein of coronaviruses to cellular receptors, which facilitates virus attachment to the surface of cells; (2) the requirement of S protein priming by cellular proteases, which undertakes S protein cleavage to fuse cell membranes and virus.[34,35] The present studies indicated that SARS-Cov-2 employs angiotensin-converting enzyme 2 (ACE2) as the cellular receptors and requires the cellular serine protease transmembrane serine protease 2 (TMPRSS2) for S protein priming.[36–41]

ACE2 is a type I transmembrane protein that was first discovered in humans as a homologous of ACE.[42–44] ACE2 has two domains, namely peptidase_M2 domain in N-terminus and collectrin domain in C-terminus.[45] Evidence shows that human ACE2 is widely distributed in several tissues, including the heart, kidney, lung, liver, testis, and intestine.[43] Biological functions of ACE2 could be divided into two categories: peptidase-dependent and -independent. The peptidase-independent function of ACE2 refers to a receptor of SARS-CoV-2 for cell entry.[45] TMPRSS2 is a type II transmembrane serine protease that was first identified in prostate cancer.[46] TMPRSS2 has three domains: Low-density lipoprotein receptor domain class A (LDLa), scavenger receptor cysteine-rich domain (SR), and trypsin-like serine protease (Tryp_SPc) domain. TMPRSS2 is widely expressed in the prostate, intestine, stomach, liver, lung, and kidney and so on.[47]

In this study, we performed an extensive BLASTP search to identify *ACE2* and *TMPRSS2* gene homologs in lower vertebrate classes (cyclostomata, fishes, amphibians, reptiles, birds,) and each order and family in mammals. We found *ACE2* and *TMPRSS2* originated from vertebrates, but *TMPRSS2* disappeared in some vertebrates. The phylogenetic tree of ACE2 demonstrates a structure consistent with a topology law of species evolution and two main clades within highly advanced mammals along with SARS-CoV-2 positive case animals and cells, which is similar to those observed in the TMPRSS2 gene family. Importantly, the distribution of 25 known human ACE2 residues affinity to SARS-CoV-2 among different species was analyzed using Fisher exact test by SAS 9.4, and 6 of those residues is highly associated with viral susceptibility with $p < .01$. Subsequently, the probability of the interplay of SARS-CoV-2 with ACE2 across different species was predicted by random forest analysis for those highly associated amino acids using randomForest function from R randomForest library. To further confirm our prediction, the hydrophobicity, the prediction, and alignment of three-dimensional (3D) structure of ACE2 from seven selected species were analyzed. Compared to human ACE2, less binding of SARS-CoV-2 to ACE2 was observed in seven animals, but with a reasonable structure for viral cell entry. Overall, our study is taken to identify viral entry in potential hosts through sequence comparison, prediction of probability for infectious and homology modeling, which can provide clues to better understand the potential human-to-animals transmission of SARS-CoV-2.

2 | MATERIALS AND METHODS

2.1 | Gene extraction

All ACE, ACE2, and TMPRSS2 genes from different species present in this study were retrieved as follows: the amino acid sequences of Human ACE (Genbank number: NP_000780), Human ACE2 (Genbank number: NP_001358344) and Human TMPRSS2 (Genbank number: NP_001128571) were download from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). According to the topology law of species evolution, representative animals from mammals, aves, peptile, amphibian, teleost fishes, cyclostomata, arthropoda, and nematomorpha were selected. The ACE, ACE2, and TMPRSS2 genes from those representative animals were extracted based on the best hits using extensive BLASTP against NCBI and Ensembl (https://useast.ensembl.org/index.html) database with human ACE, ACE2, and TMPRS2 as the queries.

2.2 | Sequence analysis

Functional domains or motifs in ACE2 and TMPRSS2 genes were confirmed by SMART (http://smart.embl-heidelberg.de/). The presence of signal peptide and transmembrane regions of ACE2 and TMPRSS2 were predicted by SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/) and TMHHM Server 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The locations of N-glycosylation site were predicted by NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc).

2.3 | Evolutionary analysis

Multiple alignments of ACE, ACE2 and TMPRSS2 from representative species were performed using Clustal X. Phylogenetic

tree was constructed using Neighbor-Joining (NJ) by MEGA X package, and in each case, branch confidence values were obtained by bootstrapping with 1000 replications. Substitutions model were analyzed with *p*-distance correction, and gaps were removed by Pairwise deletion.

2.4 | Statistical analysis for prediction

The frequencies of amino acids were summarized by the status of SARS-CoV-2 infection for each interested position for the 13 species including 9 known species has positive receptor binding and 4 species has negative receptor binding. The association of amino acid with the status virus infection were tested using Fisher exact test by SAS 9.4. The heatmap of amino acids on each interested potion for the 13 species were generated using Heatmap function from R library ComplexHeatmap (https://jokergoo.github.io/ComplexHeatmap-reference/book/). To further predict the probability of the recognition of ACE2 to SARS-CoV-2 from other species, we first choose the six position that have a *p* value less than .01 from the Fisher exact test, which are Ser19, Lys26, Thr27, Asp30, Leu79, and Met82. For these positions, if the amino acids are in species which are recognized by the virus, then the amino acid will be coded as 1, if the amino acids are in species which are not recognized with the virus, then it will be coded as 0, otherwise, the amino acids will be coded as unknown. Using the transferred data, we conducted a random forest analysis for the five positions using randomForest function from R randomForest library. The 13 species are treated as training datasets and the virus infection status of the rest species are predicted accordingly.

2.5 | Protein structure analysis

The amino acid sequences of ACE2 from seven representative species were selected from the multiway alignment and loaded into GOR IV (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) for secondary protein structure, DNAMAN for the hydrophobicity of amino acids, and I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER), ranked as the Top one server in template-based protein structure modeling, for 3D structural prediction. The alignment of 3D structure was performed by TM-align (https://zhanglab.ccmb.med.umich.edu/TM-align/).[48,49]

3 | RESULTS

3.1 | Summary of animals and cells affected by SARS-CoV-2

To date, limited emergence of COVID-19 cases in animals and cells has been reported. For animals, rhesus macaques, golden hamsters, domestic cats, dogs, ferret, Egyptian fruit bat, and rabbits are susceptible to experimental infection by SARS-CoV-2. Lion and tiger were confirmed by the United States Department of Agriculture (Table S1). Viral RNA can be detected in the related tissues or cell lines in all species listed in Table S1. The clinical sign of respiratory tract was confirmed in rhesus macaques, golden hamster, domestic cats, tiger, lion, mink, and ferret; Viral transmission was observed in rhesus macaques, golden hamster, domestic cats, lion, tiger, mink, ferret, and Egyptian fruit bat.[12–23,25–29,31–33,50] For cell lines, SARS-CoV-2 can infect and replicate in human Caco2, Calu3, NHBE, and lung epithelial line A549.[25,51–56] Meanwhile, SARS-CoV-2 can replicate in rhesus monkey LLCMK2, rabbit RK-13, cat CRFK, and pig ST, and PK-15 cells lines.[25,57] Overall, the above reports revealed that the SARS-CoV-2 cell entry, or at the least, the recognition of ACE2 to SARS-CoV-2 has widely existed in livestock and wide animals. Given the reported important role of ACE2 and TMPRSS2 in SARS-CoV-2 cell entry, we subsequently carried out the bioinformatics analysis for these two genes to explore the potential clues to the identification of intermediate host and human-to-animals transmission of SARS-CoV-2.

3.2 | Sequence identification of ACE2

To define ACE2 genes in different species, amino acids of human ACE2 were used as a means to identify homologues in NCBI and Ensembl database by BLASTP. As shown in Table 1, the number of ACE2 homologous in almost all vertebrates and invertebrates is 1, only in fresh-water polyp *Hydra vulgaris* is it 2. Vertebrate ACE2 contains peptidase M2 domain and signal peptide in N-terminus, and collectrin domain and transmembrane region in C-terminus; However, collectrin domain and trans-membrane region are not present in homolog/orthologues from invertebrate fruit fly *D. melanogaster*, roundworm *C. elegans and* fresh-water polyp *H. vulgaris* (Figure S1). The absence of collectrin domain in inveterate homologous/orthologues is similar to vertebrate ACE, the orthologues of the ACE2 gene family. Consistently with the domain composition, the homology of invertebrate homolog/orthologues to human ACE is higher than human ACE2. For instance, the identity of fruit fly ACE to human ACE is 44.35%, while the identity to human ACE2 is 36.47%. To further examine the evolutionary relationship of ACE and ACE2, an NJ tree was created by MEGA X based on their amino acid sequences. As shown in Figure S1, the tree can be split into two clades, one clade contains vertebrate ACE2, and the other clade contains invertebrate homologous/orthologues and vertebrate ACE, suggesting invertebrate homologous/orthologues is closed to vertebrate ACE. Therefore, the earliest of ACE2 occurs invertebrate.

The cryo-electron microscopy structures of the full-length human ACE2 indicated that the extracellular region is highly glycosylated, and Asn90 was confirmed to prevent the binding of ACE2 and SARS-CoV-2.[58] Based on some experimental evidence and bioinformatics predictions,[59] the number of N-glycosylation sites appears to be varied across different species as following (Table 1). Firstly, the number of glycosylation sites is different in the range from 1 in very lowly advanced vertebrates (zebrafish and sea lamprey) to 8 in

TABLE 1 ACE1 and ACE2 genes from representative animals

Gene	Species	Common name	Accession number	Identities to human ACE2 (%)	Length (aa)	Position of peptidase M2 domain 1	Position of peptidase M2 domain 2	Position of collectrin domain	Signal peptide	Trans-membrane region	N-Glycosylation sites
ACE2	*H. sapiens*	human	NP_001358344.1	100	805	19–606	N	617–770	1–17	741–763	53, 90, 103, 322, 432, 546, 690
	M. mulatta	Rhesus macaque	NP_001129168.1	94.96	805	19–606	N	617–770	1–17	740–762	53, 90, 103, 322, 432, 546, 690
	M. fascicularis	Crab-eating macaque	XP_005593094.1	95.21	805	19–606	N	617–770	1–17	740–762	53, 90, 103, 322, 432, 546, 690
	M. auratus	Golden hamster	XP_005074266.1	84.26	805	19–606	N	617–770	1–17	740–762	53, 82, 90, 432, 658, 690, 728, 772
	M. musculus	Mouse	NP_001123985.1	81.86	805	19–606	N	617–770	1–17	740–762	53, 536, 546, 660, 690, 772
	C. porcellus	Guinea pig	XP_023417808.1	77.27	813	27–614	N	625–778	1–25	748–770	61, 98, 224, 440, 698, 809
	O. cuniculus	Rabbit	QHX39726.1	84.76	805	19–606	N	617–770	1–17	741–763	53, 90, 134, 432
	F. catus	Cat	NP_001034545.1	85.22	805	19–606	N	617–770	1–17	740–762	53, 90, 216, 299, 322, 690
	P. concolor	Cougar	XP_025790417.1	85.59	805	19–606	N	617–770	1–17	740–762	53, 90, 216, 299, 322, 690
	P. tigris altaica	Tiger	XP_007090142.1	85.70	797 (partial)	11–598	N	609–762	1–13	732–754	45, 82, 208, 291, 314, 682
	C. crocuta	Spotted hyena	KAF0878287.1	83.35	805	19–606	N	617–770	1–17	740–762	53, 216, 322, 690
	S. suricatta	Meerket	XP_029786256.1	82.86	805	19–606	N	617–770	1–17	740–762	53, 90, 216, 690
	P. larvata	Masked palm civet	Q56NL1.1	83.48	805	19–606	N	617–770	1–17	740–762	53, 216, 322, 432, 690
	U. arctos horribilis	Grizzly bear	XP_026333865.1	83.88	805	19–606	N	617–770	1–17	740–762	53, 90, 216, 322, 432, 690
	U. maritimus	Polar bear	XP_008694637.1	83.92	790 (partial)	4–591	N	602–755	1–31	725–747	38, 75, 201, 307, 417, 675
	A. melanoleuca	Panda	EFB23904.1	83.38	805	19–606	N	617–770	1–17	740–762	53, 90, 216, 322, 432, 690
	P. lotor	Raccoon	BAE72462.1	83.88	805	19–606	N	617–770	1–17	740–762	34, 53, 216, 322, 432, 690

TABLE 1 (Continued)

Gene	Species	Common name	Accession number	Identities to human ACE2 (%)	Length (aa)	Position of peptidase M2 domain 1	Position of peptidase M2 domain 2	Position of collectrin domain	Signal peptide	Trans-membrane region	N-Glycosylation sites
	M. putorius furo	Ferret	NP_001297119.1	82.74	805	19–606	N	617–770	1–17	740–762	53, 322, 690
	C. lupus dingo	Dingo (Dog)	XP_025292925.1	84.01	804	18–605	N	616–769	1–17	739–761	53, 133, 215, 298, 321, 689
	C. lupus familiaris	Dog	NP_001158732.1	83.50	804	18–605	N	616–769	1–17	739–761	53, 133, 215, 298, 321, 681, 689
	N. procyonoides	Raccoon dog	ABW16956.1	84.01	804	18–605	N	616–769	1–17	739–761	53, 133, 215, 298, 321, 689
	V. vulpes	Red fox	XP_025842512.1	83.63	804	18–605	N	616–769	1–17	739–761	53, 133, 215, 298, 321, 689
	M. javanica	Malayan pangolin	XP_017505752.1	84.76	805	19–606	N	617–770	1–17	740–762	53, 90, 216, 299, 432, 690
	O. aries	Sheep	XP_011961657.1	81.74	804	19–605	N	616–769	1–17	739–761	53, 90, 431, 545, 659, 689
	C. hircus	Goat	NP_001277036.1	81.74	804	19-605	N	616–769	1–17	739–761	53, 90, 431, 545, 659, 689
	B. taurus	Taurine cattle	XP_005228486.1	81.12	804	19–605	N	616–769	1–17	739–761	53, 90, 298, 431, 545, 659, 689
	B. mutus	Wild Yak	XP_005903173.1	81.37	804	19–605	N	616–769	1–17	739–761	53, 90, 298, 431, 545, 659, 689
	B. indicus	Humped cattle	XP_019811720.1	81.12	804	19–605	N	616–769	1–17	739–761	53, 90, 298, 431, 545, 689
	M. muntjak	Barking deer	KAB0345583.1	81.61	804	19–605	N	616–769	1–17	739–761	53, 90, 298, 431, 545, 659, 689
	S. scrofa domesticus	Pig	ACT66265.1	81.94	787 (partial)	19–606	N	617–770	1–17	740–762	53, 299, 322, 690
	S. scrofa	Wide pig	XP_020935033.1	81.74	805	19–606	N	617–770	1–17	740–762	53, 299, 322, 660, 690
	C. ferus	Camel	XP_006194263.1	82.23	805	19–606	N	617–770	1–17	739–761	53, 90, 322, 660, 690
	E. caballus	Horse	XP_001490241.1	86.78	805	19–606	N	617–770	1–17	739–761	53, 90, 134, 257, 322, 660, 690

(Continues)

TABLE 1 (Continued)

Gene	Species	Common name	Accession number	Identities to human ACE2 (%)	Length (aa)	Position of peptidase M2 domain 1	Position of peptidase M2 domain 2	Position of collectrin domain	Signal peptide	Trans-membrane region	N-Glycosylation sites
	H. armiger	Great Roundleaf Bat	XP_019522954.1	80.38	806	19–606	N	618–771	1–17	741–763	53, 90, 322, 432, 661
	R. ferrume-quinum	greater horseshoe bat	XP_032963186.1	81.36	805	19–606	N	617–770	1–17	740–762	38, 53, 82, 90, 322, 432, 546, 690
	T. chinensis	Chinese tree shrew	XP_006164754.1	80.75	805	19–606	N	617–770	1–17	740–762	53, 78, 218, 257, 322, 690
	D. novemcinctus	Nine-banded armadillo	XP_004449124.1	79.13	804	18–605	N	616–769	1–19	739–761	53, 81, 89, 217, 321, 545
	E. europaeus	Hedgehog	XP_007538670.1	79.01	804	19–606	N	617–769	1–17	739–761	52, 81, 89, 217, 321, 545
	L. africana	Elephant	XP_023410960.1	80.50	800	19–601	N	612–765	1–17	735–757	38, 53, 90, 103, 154, 216, 432
	T. manatus latirostris	Sea Cow	XP_004386381.1	81.49	800	19–601	N	612–765	1–17	735–757	53, 154, 252, 298, 685
	O. afer afer	Aardvark	XP_007951028.1	79.38	799	19–600	N	611–764	1–17	734–756	38, 53, 90, 153, 297, 488, 601
	E. edwardii	Cape elephant shrew	XP_006892457.1	77.52	798	19–601	N	612–763	1–17	734–756	53, 154, 398, 317, 427, 683
	P. cinereus	Koala	XP_020863153.1	71.48	807	19–606	N	617–769	1–17	739–761	53, 78, 216, 322, 660
	O. anatinus	Platypus	XP_001515597.2	68.02	806	18–605	N	621–773	1–17	743–765	52, 78, 83, 321, 678, 790
	G. gallus	Chicken	XP_416822.2	65.97	808	18–606	N	617–770	1–17	741–763	52, 78, 194, 257, 280, 322, 534, 690
	A. platyrhynchos	Duck	XP_012949915.2	64.81	805	18–606	N	617–767	1–17	738–760	52, 78, 322, 534, 634, 687
	A. forsteri	Penguin	XP_009275140.1	66.75	809	18–606	N	617–770	1–17	741–763	37, 52, 78, 257, 534, 572, 690
	P. bivittatus	Snake	XP_007431942.2	61.77	827	43–628	N	639–793	1–42	763–785	77, 103

TABLE 1 (Continued)

Gene	Species	Common name	Accession number	Identities to human ACE2 (%)	Length (aa)	Position of peptidase M2 domain 1	Position of peptidase M2 domain 2	Position of collectrin domain	Signal peptide	Trans-membrane region	N-Glycosylation sites
	X. tropicalis	Frog	XP_002938293.2	60.03	862	20–610	N	622–773	1–17	744–766	54, 76
	D. rerio	Zebrafish	NP_001007298.1	57.83	785	18–607	N	630–783	1–17	752–774	545
	P. marinus	Sea lamprey	XP_032835032.1	56.39	849	19–579	N	590–742	1–15	711–733	88
Gene	*Species*	Common Name	Accession number	Identities to human ACE2 (%)	Identities to human ACE (%)	Length (aa)	Position of peptidase M2 domain-1	Position of peptidase M2 domain-2	Position of collectrin domain	Signal peptide	Trans-membrane region
ACE	*H. sapiens*	human	NP_000780.1		100	1306	40–623	643–1221	N	1–29	1257–1276
	G. gallus	Chicken	NP_001161204.1		68.03	1281	28–609	629–1207	N	1–17	1240–1262
	A. carolinensis	Green Alone	XP_008111340.1		66.88	1305	52–635	655–1233	N	1–40	1264–1286
	X. tropicalis	Frog	NP_001116882.1		65.6	1284	28–611	631–1209	N	1–16	1244–1266
	D. rerio	Zebrafish	XP_694336.5		65.18	1324	69–651	671–1249	N	1–58	1283–1305
	M. musculus	Mouse	NP_997507.1		83.08	1312	45–628	648–1226	N	1–29	1264–1286
	P. marinus	Sea lamprey	XP_032807619.1		62.47	1281	32–615	635–1212	N	1–20	1248–1274
	P. marinus-like	Sea lamprey	XP_032821231.1		60.59	1290	38–623	643–1220	N	1–27	1252–1274
	D. melanogaster	Fruit fly	AAB02171.1	36.47	44.35	615	19–606	N	N	1–17	N
	C. elegans	Roundworm	NP_001024453.1	26.59	28.01	906	175–764	N	N	1–19	N
	H. vulgaris	Hydra vulgaris	XP_004208490.1	38.63	43.15	639	20–607	N	N	1–19	N
	H. vulgaris	Hydra vulgaris	XP_012555870.1	38.15	40.65	616	21–605	N	N	1–23	N

Note: "N" means not found.

mammals (chicken, bat, and golden hamster). Human, monkey, dog, cattle, horse, aardvark, elephant, and penguin have 7 sites, while other animals have 2-6. Secondly, the position of N-glycosylation sites is varied. Asn53 (50/51) is the highest conserved sites among different species and then subsequently is Asn690 (39/51), Asn322 (29/51), Asn90 (28/51), Asn432(18/51), Asn546 (10/51), and Asn103 (3/51), whereas Asn103 only appears in primates.

3.3 | Sequence identification of TMPRSS2

A blast search identified TMPRSS2 homologous in representative organisms (Table 2), including 1 homolog in mammals, aves, reptiles, and teleost fishes, 10 homologous in frog *Xenopus tropicalis*, but not in lower vertebrate sea lamprey *Petromyzon marinus* (cyclostomata) and invertebrates, such as fruit fly *D. melanogaster* (arthropoda) and roundworm *C. elegans* (nematomorpha). Interestingly, possibly due to the incompleteness of the genome sequencing or lineage-specific gene loss/evolution in vertebrates, masked palm civet *Paguma larvata*, raccoon dog *Nyctereutes procyonoides*, pig *Sus scrofa domesticus*, and cape elephant shrew *Elephantulus edwardii* lacked Tmprss2 genes.

Compared to the homology of human ACE2 with other species, the homology between human TMPRSS2 and other species is much lower. For example, in rhesus macaque, *Macaca mulatta*, an experimental model widely used in SARS-CoV-2 research, the homology of ACE2 with humans is 94.96%, while the homology of TMPRSS2 with humans is 88.06%. Vertebrate TMPRSS2 is composed of three domains: low-density LDLa for calcium binding, SR for the binding to other cell surface or extracellular molecules, and Tryp_SPc for cleaving peptide bonds after lysine or arginine residues.[60] Interestingly, LDLa domain is not represented in ferret *Mustela putorius furo*, hedgehog *Erinaceus europaeus* and sea cow *Trichechus manatus*; *X. tropicalis* tmprss 2.2, 2.4, 2.5, 2.6, 2.7, 2.14, and 2.15 contains many (≥2) LDLa. SR domain is not identified in cougar *Puma concolor* and frog tmprss 2.4. Conversely, Tryp_SPc domain was conserved in all representative species except frog tmprss 2.4. To date, all characterized vertebrate TMPRSS2 has a signal peptide and transmembrane region. However, hedgehog lacked signal peptide, while ferret and great roundleaf bat *Hipposideros armiger* and frog tmprss 2.5 lacked trans-membrane region.

3.4 | Phylogenetic analysis of ACE2 and TMPRSS2 gene family

To examine the evolutionary relationship of ACE2 gene family, a NJ tree with bootstrapping branch confidence was constructed with MEGA X using ACE2 homologs from vertebrates, including mammals, birds, reptiles, amphibians, fishes, and cyclostomata (Figure 1). Entirely, the tree is consistent with the topology law of species evolution. The fishes (*D. rerio*) and cyclostomata (*P. marinus*) locate at the base, suggesting they are likely the ancestors of the ACE2 gene family. In addition to ACE2 from platypus *Omithorhynchus anatinus* (prototheria) and koala *Plethodon cinereus* (Metatheria) located outside the branches of vertebrate ACE2, ACE2 homologous from eutheria clusters together. In most of Eutheria, exceptt for cape elephant shrew *E. edwardii* (macroscelidea), aardvark *Orycteropus afer afer* (tubulidentata), sea Cow *T. manatus latirostris* (sirenia), elephant *Loxodonta africana* (proboscidea), hedgehog *E. europaeus* (insectivora), nine-banded armadillo *Dasypus novemcinctus* (edentata), and tupaia *Tupaia chinensis* (scandentia), ACE2 splits into two main branches: one branch contains species from primates, rodentia and lagomorpha; the other branch contains species from carnivora, pholidota, artiodactyla, perissodactyla, and chiroptera.

Moreover, an NJ tree of TMPRSS2 from different species was created to detect its evolutionary relationship. Entirely, the TMPRSS2 tree is similar to ACE2 tree, consistently with the species phylogeny (Figure 1). In addition to frog tmprss2.1, other 9 frog tmprss2 genes situ in the root of the tree, indicating a lineage-specific duplication or gene conversion to produce massive homologs occurs in frogs. Compared to the ACE2 tree, hedgehog *E. europaeus* (insectivora) in TMPRSS2 tree locates at the base of vertebrates, while tupaia *T. chinensis* (scandentia) clusters closely to primates. Given the reported cases of animals or cell lines (Table S1) that appear in both branches 1 and 2, it is therefore possible that animals from those two branches is susceptible to SARS-CoV-2 infections.

3.5 | Comparison of interacting amino acids of ACE2 and SARS-CoV-2

Cocrystallization and the structural determination of SARS-CoV-2 S protein with full-length human ACE2 identified a total of 25 amino acids in ACE2 was critical for its binding to SARS-CoV-2.[58,61–65] To confirm the conservation of these 25 residues across different species, the amino acid sequence alignments by Clustal X were performed. As shown in Table 3, Gly352, Gly355, and Gly357 were conserved among all vertebrate ACE2; Phe28 (50/51), Arg393 (50/51), Leu45 (47/51), and Asn330 (48/51) is highly conserved across all vertebrates; Met82 only presents in primates. To identify which residues are highly associated with the recognition of ACE2 to SARS-CoV-2, we performed Fisher exact test by SAS 9.4. Based on the receptor recognition summarized in Table S1, we selected nine species as the positive samples and four lower advanced species including snake, frog, fish, and sea lamprey, as the negative samples. We observed the Phe28, Asp355, Arg357, and Arg93 are present in both negative and positive animals, indicating that they might be not relevant to the recognition of ACE2 to SARS-CoV-2 among different species (Table S2). Nevertheless, the *p-value* of His34, Glu35, Asp38, Tyr41, Gln42, Leu45, Asn330, and Gly354 is above .05, whereas other residues are under .05 (Figure S2). In particular, the *p-value* of Ser19, Lys26, Thr27, Asp30, Leu79, and Met82 is lower than .01 (Figure 2A), implying those amino acids are highly associated with the binding of ACE2 to SARS-CoV-2.

TABLE 2 TMPRSS2 gene from representative animals

Species	Common Name	Accession number	Identities to human (%)	Length (aa)	Position of LDLa domain (low-density lipoprotein receptor domain class A)	Position of SR domain (Scavenger receptor Cys-rich)	Position of Tryp_SPc domain (Trypsin-like serine protease)	Signal peptide	Trans-membrane region
H. sapiens	Human	NP_001128571.1	100	529	149–187	186–279	292–521	1–49	121–143
M. mulatta	Rhesus macaque	XP_028701148.1	88.06	534	154–192	191–284	297–526	1–29	126–148
M. fascicularis	Crab-eating macaque	XP_005548700.1	86.6	525	145–183	182–275	288–517	1–46	117–139
M. auratus	Golden hamster	XP_012971684.1	76.67	491	112–150	149–242	254–483	1–13	85–107
M. musculus	Mouse	NP_056590.2	78.41	490	111–149	148–241	253–482	1–13	86–108
C. porcellus	Guinea pig	XP_013001524.1	67.82	523	109–147	146–239	251–480	1–38	83–105
O. cuniculus	Rabbit	XP_008250697.1	74.22	511	135–171	170–262	274–503	1–36	107–129
F. catus	Cat	XP_023094477.1	79.67	492	112–150	149–242	255–484	1–13	84–106
P. concolor	Cougar	XP_025768873.1	58.42	446	112–150	N	212–402	1–13	84–106
P. tigris altaica	Tiger	XP_015396688.1	69.11	518	112–150	149–242	224–510	1–13	84–106
C. crocuta	Spotted hyena	KAF0886132.1	79.38	492	112–150	149–242	255–484	1–50	84–106
S. suricatta	Meerket	XP_029794796.1	77.8	492	112–150	149–242	255–484	1–50	84–106
P. larvata	Masked palm civet	N							
U. arctos horribilis	Grizzly bear	XP_026355755.1	79.43	492	112–150	149–242	255–484	1–41	84–106
U. maritimus	Polar bear	XP_008708425.1	78.86	524	112–150	149–242	255–487	1–41	84–106
A. melanoleuca	Panda	XP_034511296.1	78.41	492	112-–150	149–242	255–484	1–41	84–106
P. lotor	Raccoon	N							
M. putorius furo	Ferret	XP_012916721.1	72.89	551	N	236–301	314–543	1–46	N
C. lupus dingo	Dingo (Dog)	XP_025317827.1	80.24	492	112–150	149–242	255–484	1–13	84–106
C. lupus familiaris	Dog	BBD33861.1	80.04	492	112–150	149–242	255–484	1–13	84–106
N. procyonoides	Raccoon dog	N							
V. vulpes	Red fox	XP_025839165.1	79.84	492	112–150	149–242	255–484	1–13	84–106
M. javanica	Malayan pangolin	XP_017508124.1	78.16	527	112–150	149–242	255–484	1–50	84–106
O. aries	Sheep	XP_027816505.1	79.02	490	112–148	147–240	253–482	1–13	84–106

(Continues)

TABLE 2 (Continued)

Species	Common Name	Accession number	Identities to human (%)	Length (aa)	Position of LDLa domain (low-density lipoprotein receptor domain class A)	Position of SR domain (Scavenger receptor Cys-rich)	Position of Tryp_SPc domain (Trypsin-like serine protease)	Signal peptide	Trans-membrane region
C. hircus	Goat	XP_005675686.1	77.39	490	112–148	147–240	253–482	1–13	84–106
B. taurus	Taurine cattle	NP_001075054.1	78.41	490	112–148	147–240	253–482	113	84–106
B. mutus	Wild Yak	XP_005893081.1	78.21	490	112–148	147–240	253–482	1–13	84–106
B. indicus	Humped cattle	XP_019818444.1	78.41	490	112–148	147–240	253–482	1–13	84–106
M. muntjak	Barking deer	KAB0356950.1	77	503	110–146	145–238	251–480	1–45	82–104
S. scrofa domesticus	Pig	N							
S. scrofa	Wide pig	BAF76737.1	76.92	495	114–152	151–244	258–487	1–13	86–108
C. ferus	Camel	XP_032317729.1	77.6	492	112–150	149–242	255–484	1–13	84–106
E. caballus	Horse	XP_005606217.1	74.75	490	112–148	147–240	253–482	1–13	84–106
H. armiger	Great Roundleaf Bat	XP_019481585.1	84.33	384	4–42	41–134	147–376	1–27	N
R. ferrumequinum	greater horseshoe bat	XP_032944694.1	80.86	492	112–150	149–242	255–484	1–13	84–106
T. chinensis	Chinese tree shrew	XP_027630699.1	70.92	500	112–148	147–240	253–492	1–13	84–106
D. novemcinctus	Nine-banded armadillo	XP_004466361.1	79.32	460	80–118	117–210	223–452	1–44	52–74
E. europaeus	Hedgehog	XP_016049831.1	68.91	479 (partial)	N	152–236	243–472	N	17–39
L. africana	Elephant	XP_023414879.1	74.49	497	111–149	148–241	254–483	1–13	84–106
T. manatus latirostris	Sea Cow	XP_023594890.1	69.82	491	N	148–241	254–483	1–38	81–103
O. afer afer	Aardvark	XP_007945507.1	78.94	524	112–150	149–242	255–484	1–13	84–106
E. edwardii	Cape elephant shrew	N							
P. cinereus	Koala	XP_020831920.1	69.98	492	111–149	148–242	255–484	1–48	83–105

TABLE 2 (Continued)

Species	Common Name	Accession number	Identities to human (%)	Length (aa)	Position of LDLa domain (low-density lipoprotein receptor domain class A)	Position of SR domain (Scavenger receptor Cys-rich)	Position of Tryp_SPc domain (Trypsin-like serine protease)	Signal peptide	Trans-membrane region
O. anatinus	Platypus	XP_028902467.1	71.49	492	111–149	148–242	255–484	1–57	83–105
G. gallus	Chicken	XP_015156666.1	59.34	486	104–142	141–235	249–478	1–53	77–99
A. platyrhynchos	Duck	XP_021132301.2	56.22	482	101–138	137–231	245–474	1–61	75–97
A. forsteri	Penguin	XP_009281370.1	58.51	484	102–140	139–233	247–476	1–49	76–98
P. bivittatus	Snake	XP_007441832.1	57.26	487	105–143	142–236	250–479	1–54	78–100
X. tropicalis	Frog	XP_004912265.1	48.47	482	110–145	148–241	247–474	1–59	82–104
		XP_004912262.1	51.73	521	101–136, 141–176	175–269	281–513	1–60	67–89
		XP_031752391.1	28.57	571	142–177, 203–241, 267–305, 338–376, 398–436, 441–476, 483–518, 525–560	N	N	1–34	106–128
		XP_031752394.1	50.67	497	2–33, 38–73, 78–113, 120–155	154–248	260–489	1–18	N
		XP_002936535.2	51.21	695	107–142, 149–184, 193–228, 235–270, 277–312, 319–354	353–447	459–687	1–60	72–94
		XP_031752397.1	50	511	94–129, 130–166	165–259	271–503	1–41	67–89
		XP_031752400.1	45.72	497	122–157	156–249	260–489	1–37	96–108
		XP_002943001.1	45.52	460	64–99	121–216	221–452	1–47	35–57
		XP_031752402.1	50.13	504	88–123, 126–161	160–253	264–495	1–23	55–77
		XP_031752206.1	43.65	504	88–123, 126–161	160–253	264–495	1–23	55–77
D. rerio	Zebrafish	NP_001008623.1	44.4	486	133–170	173–224	251–479	1–39	107–129
P. marinus	Sea lamprey	N							
D. melanogaster	Fruit fly	N							
C. elegans	Roundworm	N							

Note: "N" means not found.

Abbreviations: LDLa, lipoprotein receptor domain class A; SR, scavenger receptor cysteine-rich domain; Tryp_SPc, trypsin-like serine protease.

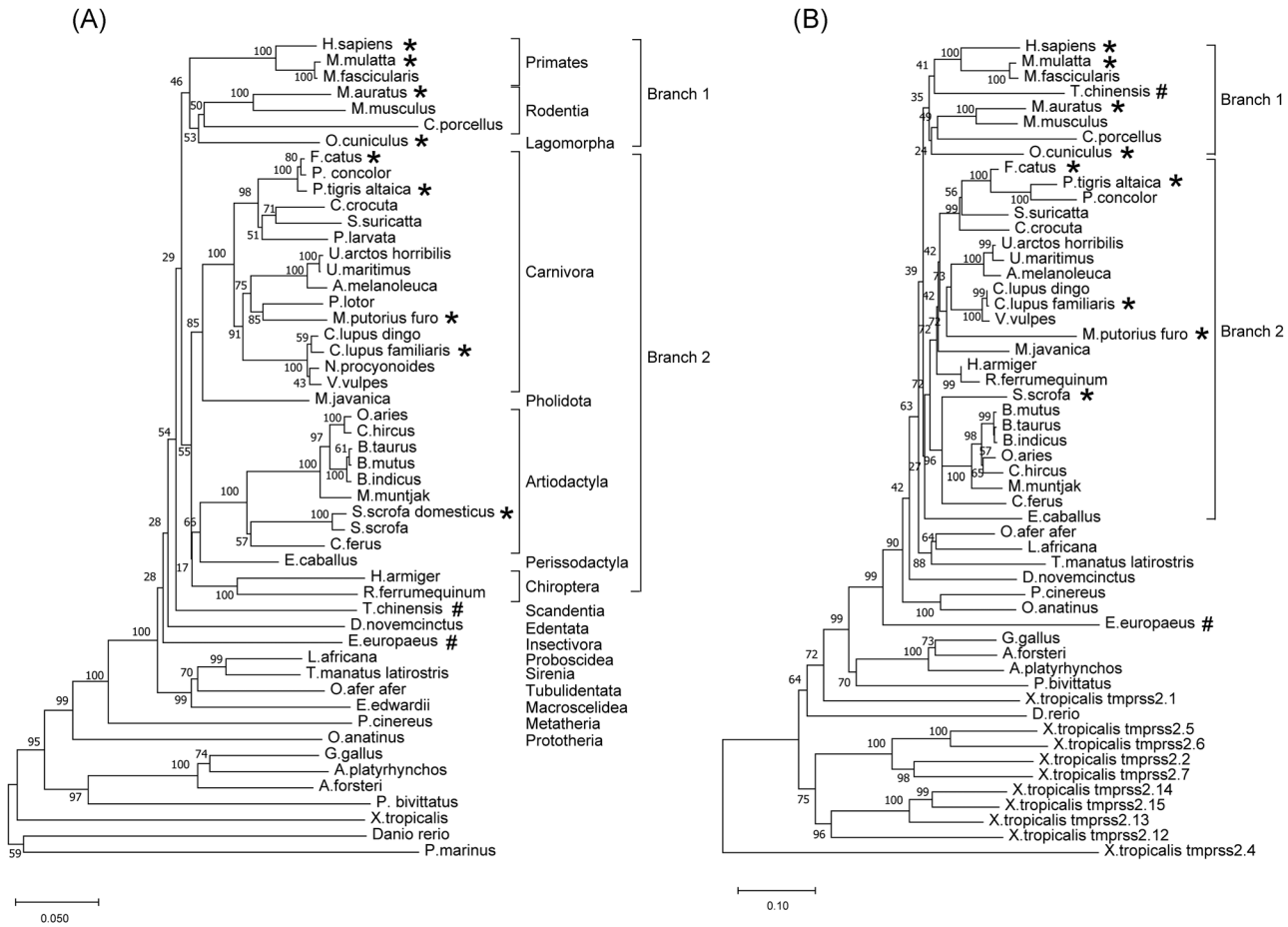


FIGURE 1 Neighbor-joining phylogenetic tree of ACE2 (left) and TMPRSS2 (right) gene family. The confidence values of bootstrap derived from 1000 replications, and the percentage of bootstrap is shown on interior branches. The scale bar shows the number of substitutions per site. (A) ACE2 tree. (B) TMPRSS2 tree. *X. tropicalis* tmprss2 gene is shown as gene symbols from Genbank to species names distinguish different tmprss2 homologous. The gene accession numbers used in this figure refer to Tables 1 and 2. Entirely, both ACE2 and TMPRSS2 gene from different species clusters as species taxonomy, which is labeled next to the tree. ACE2 and TMPRSS2 genes from the highly advanced animals could be split into two branches (Branches 1 and 2). *Labeled the animals or cells which has been confirmed to be recognized by SARS-CoV-2. #Labeled the species whose position is quite different between ACE2 and TMPRSS2 trees. ACE2, angiotensin-converting enzyme 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

To further dissect the probability of the recognition of ACE2 to SARS-CoV-2 in other unknown species, a random forest analysis using the randomForest function from the R randomForest library was performed. As Leu79 has too many species with unknown status of amino acids, only Ser19, Lys26, Thr27, Asp30, and Met82 were used for this analysis. The identified receptor recognition shown in "Yes" in Figure 2B is mainly distributed in two classes, carnivora and artiodactyla, and contains horse, malayan pangolin, and sea cow. These identified species comprise wide animals and many important economy livestock, such as sheep and cows.

3.6 | Structure analysis of ACE2 gene family

To further explore the recognition of ACE2 to SARS-CoV-2 across different species, we selected ACE2 genes from 7 animals according to the reported cases in Table S1 (rabbit and pig), analysis on Figure 2 (the "Yes" for horse, cattle, and malayan pangolin; "NA" for platypus), and snake for negative binding and carried out their structure analysis. It is known that the networks of hydrophobic interactions and hydrogen-bonding within the interface between ACE2 and the receptor-binding domain (RBD) of SARS-CoV-2 could enhance this receptor binding.[61] The hydrophobic contact of Phe486 of RBD with ACE2 is situated in a pocket fenced by Leu79, Met82, and Tyr83 at the N-terminal end of ACE2. Indeed, Tyr83 contributes a hydrogen bond to Asn487 of the RBD.[61] Therefore, we first analyzed the hydrophobicity of the amino acids of 76 to 85 among various species by DNAMAN. As per Figure 3A, compared with the hydrophobicity value of human ACE2 above zero, the hydrophobicity value within the other seven animals is below zero, indicating the key part of ACE2 from the other seven species is not hydrophobic. Given the enhancement of hydrophobic contact on the binding of ACE2 and

TABLE 3 Comparison of ACE2 residues binding to SARS-CoV-2 spike from different species

Common Name	Receptor binding	SER 19	GLN 24	LYS 26	THR 27	PHE 28	ASP 30	LYS 31	HIS 34	GLU 35	GLU 37	ASP 38	TYR 41	GLN 42	LEU 45	LEU 79	MET 82	TYR 83	ASN 90	ASN 330	GLY 352	LYS 353	GLY 354	ASP 355	ARG 357	ARG 393	similarity to human
human	+	SER	GLN	LYS	THR	PHE	ASP	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	LEU	MET	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	25
Rhesus macaque	+	SER	GLN	LYS	THR	PHE	ASP	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	LEU	MET	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	25
Crab-eating macaque		SER	GLN	LYS	THR	PHE	ASP	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	LEU	MET	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	25
Golden hamster	+	SER	GLN	LYS	THR	PHE	ASP	LYS	GLN	GLU	GLU	ASP	TYR	GLN	LEU	LEU	ASN	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	23
Mouse		SER	ASN	LYS	THR	PHE	ASN	ASN	GLN	GLU	GLU	ASP	TYR	GLN	LEU	THR	SER	PHE	THR	ASN	GLY	HIS	GLY	ASP	ARG	ARG	16
Guinea pig		PHE	GLN	LYS	THR	PHE	ASP	GLU	LEU	LYS	GLU	ASP	TYR	GLN	LEU	LEU	ALA	TYR	ASN	ASN	GLY	LYS	ASN	ASP	ARG	ARG	19
Rabbit	+	SER	LEU	LYS	THR	PHE	GLU	LYS	GLN	GLU	GLU	ASP	TYR	GLN	LEU	LEU	THR	TYR	ASN	ASN	GLY	LYS	ARG	ASP	ARG	ARG	20
Cat	+	SER	LEU	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	21
Cougar		SER	LEU	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	21
Tiger	+	SER	LEU	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	21
Spotted hyena		SER	LEU	LYS	THR	PHE	GLU	LYS	TYR	GLU	GLN	GLU	TYR	LEU	LEU	LEU	THR	TYR	ASP	ASN	GLY	LYS	GLY	ASP	ARG	LYS	16
Meerket		SER	LEU	LYS	THR	PHE	GLU	GLN	HIS	GLU	GLN	GLU	TYR	LEU	VAL	ARG	ALA	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	16
Masked palm civet		SER	LEU	LYS	THR	PHE	GLU	THR	TYR	GLU	GLN	GLU	TYR	GLN	VAL	LEU	THR	TYR	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	16
Grizzly bear		SER	LEU	GLU	THR	PHE	GLU	LYS	TYR	GLU	GLU	ASP	TYR	GLN	LEU	HIS	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Polar bear		SER	LEU	GLU	THR	PHE	GLU	LYS	TYR	GLU	GLU	ASP	TYR	GLN	LEU	HIS	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Panda		SER	LEU	GLU	THR	PHE	GLU	LYS	TYR	GLU	GLU	ASP	TYR	GLN	LEU	HIS	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Raccoon		SER	LEU	ASN	THR	PHE	GLU	ASN	ASN	GLU	GLU	GLU	TYR	GLN	LEU	GLN	THR	TYR	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	18
Ferret	+	SER	LEU	LYS	THR	PHE	GLU	LYS	TYR	GLU	GLU	GLU	TYR	GLN	LEU	HIS	THR	TYR	ASP	ASN	GLY	LYS	ARG	ASP	ARG	ARG	20
Dingo (Dog)		SER	LEU	LYS	THR	PHE	GLU	LYS	TYR	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	20
Dog	+	SER	LEU	LYS	THR	PHE	GLU	LYS	TYR	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Raccoon dog		SER	LEU	ASN	THR	PHE	GLU	LYS	TYR	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASP	ASN	GLY	ARG	GLY	ASP	ARG	ARG	17
Red fox		SER	LEU	ASN	THR	PHE	GLU	LYS	TYR	GLU	GLU	GLU	TYR	GLN	LEU	LEU	THR	TYR	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	18
Malayan pangolin		SER	GLU	LYS	THR	PHE	GLU	LYS	SER	GLU	GLU	GLU	TYR	GLN	LEU	ILE	ASN	TYR	ASN	ASN	GLY	LYS	HIS	ASP	ARG	ARG	18
Sheep		SER	GLN	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	MET	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	22
Goat		SER	GLN	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	MET	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	22
Taurine cattle		SER	GLN	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	MET	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	22
Wild Yak		SER	GLN	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	MET	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	22
Humped cattle		SER	GLN	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	MET	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	22
Barking deer		SER	GLN	LYS	THR	PHE	GLU	LYS	HIS	GLU	GLU	ASP	TYR	GLN	LEU	MET	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	22
Pig	+	SER	LEU	LYS	THR	PHE	GLU	LYS	LEU	GLU	GLU	ASP	TYR	GLN	LEU	ILE	THR	TYR	THR	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Wide pig		SER	LEU	LYS	THR	PHE	GLU	LYS	LEU	GLU	GLU	ASP	TYR	GLN	LEU	ILE	THR	TYR	THR	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Camel		SER	LEU	LYS	THR	PHE	GLU	GLU	HIS	GLU	GLU	ASP	TYR	GLN	LEU	THR	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	20
Horse		SER	LEU	LYS	THR	PHE	GLU	LYS	SER	GLU	GLU	GLU	HIS	GLN	LEU	LEU	THR	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	19
Great Roundleaf Bat		SER	LEU	LYS	GLU	PHE	ASP	LYS	THR	GLU	GLU	ASP	HIS	LEU	LEU	ARG	ASP	TYR	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	18
greater horseshoe bat		SER	LEU	LYS	LYS	PHE	ASP	ASP	SER	GLU	GLU	ASN	HIS	GLN	LEU	LEU	ASN	PHE	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	17
Chinese tree shrew		THR	GLU	LYS	VAL	PHE	ASN	LYS	ILE	GLU	GLU	GLU	HIS	GLN	LEU	GLN	ARG	TYR	ASP	LYS	GLY	LYS	ASN	ASP	ARG	ARG	13
Nine-banded armadillo		SER	GLN	SER	THR	PHE	GLU	THR	GLN	GLN	GLU	GLU	HIS	GLN	LEU	MET	ASN	PHE	ASN	ASN	GLY	LYS	GLY	ASP	ARG	ARG	15
Hedgehog		THR	GLU	LYS	LYS	PHE	ASP	ASP	ARG	GLN	GLU	ASN	TYR	GLU	LEU	THR	ASN	TYR	ASN	ASN	GLY	ASN	GLY	ASP	ARG	ARG	14
Elephant		SER	LEU	ARG	THR	PHE	ASP	THR	GLN	GLU	GLU	ASP	TYR	GLN	LEU	LEU	ASP	PHE	SER	ASN	GLY	LYS	GLY	ASP	ARG	ARG	18
Sea Cow		SER	LEU	ARG	THR	PHE	ASP	THR	GLN	GLU	GLU	ASP	TYR	GLN	LEU	LEU	ASN	PHE	SER	ASN	GLY	LYS	GLY	ASP	ARG	ARG	18
Aardvark		ALA	LEU	GLY	THR	PHE	GLU	LYS	GLN	GLU	GLU	ASN	TYR	GLN	LEU	ILE	SER	PHE	ASN	LYS	GLY	LYS	GLY	ASP	ARG	ARG	15
Cape elephant shrew		PRO	GLN	LYS	ALA	PHE	GLU	GLN	GLN	GLN	GLU	ASP	TYR	GLN	LEU	VAL	ASN	PHE	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	15
Koala		PHE	ARG	LYS	GLU	PHE	GLU	THR	LYS	GLU	GLU	GLU	TYR	GLN	LEU	ILE	THR	PHE	ASP	ASN	GLY	LYS	GLY	ASP	ARG	ARG	14
Platypus		LYS	GLU	ARG	GLN	PHE	THR	GLN	LYS	GLN	GLU	ASP	TYR	GLN	LEU	ASN	LYS	PHE	ASP	ASN	GLY	LYS	ASN	ASP	ARG	ARG	12
Chicken		ASP	GLU	GLN	THR	PHE	ALA	GLU	VAL	ARG	GLU	ASP	TYR	GLU	LEU	ASN	ARG	PHE	ASP	ASN	GLY	LYS	ASN	ASP	ARG	ARG	12
Duck		ASP	GLN	LYS	MET	PHE	ALA	GLU	VAL	ARG	GLU	ASP	TYR	GLU	LEU	ASN	ASN	PHE	ASP	ASN	GLY	LYS	ASN	ASP	ARG	ARG	13
Penguin		ASP	GLN	GLN	MET	PHE	GLU	GLU	LYS	ARG	GLU	ASN	TYR	GLU	LEU	ASN	SER	PHE	ASP	ASN	GLY	LYS	ASN	ASP	ARG	ARG	11
Snake	-	ASP	GLU	ALA	GLU	PHE	MET	GLN	VAL	ARG	ASP	ASP	TYR	ASP	ILE	ASN	LYS	PHE	ASP	ASN	GLY	LYS	LYS	ASP	ARG	ARG	9
Frog	-	GLN	GLN	ARG	ASP	PHE	LYS	ARG	GLN	GLU	GLU	VAL	HIS	GLN	LEU	ASN	ALA	PHE	ASP	ASN	GLY	MET	ASN	ASP	ARG	ARG	11
Zebrafish	-	THR	ARG	ARG	GLU	PHE	ASN	LYS	GLU	GLU	SER	ASP	TYR	GLN	LEU	GLU	ALA	TYR	ASP	ASN	GLY	ARG	LYS	ASP	ARG	ARG	13
Sea lamprey	-	-	-	-	-	-	-	-	ARG	LEU	ARG	ARG	TRP	GLU	CYS	GLU	GLN	PHE	ASP	HIS	GLY	GLY	ARG	ASP	ARG	ARG	4

Note: The 25 residues in human ACE2 interfacing with SARS-CoV-2 RBD were listed and compared for the conservation among different species. The letters in red highlight the amino acid at the corresponding positions, which has an identity to human ACE2. ACE2 sequence accession numbers used in this analysis refer to Table 2. "−" means the negative recognition of ACE2 to SARS-CoV-2; "+" means the known positive binding of ACE2 to SARS-CoV-2.
Abbreviations: ACE2, angiotensin-converting enzyme 2; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

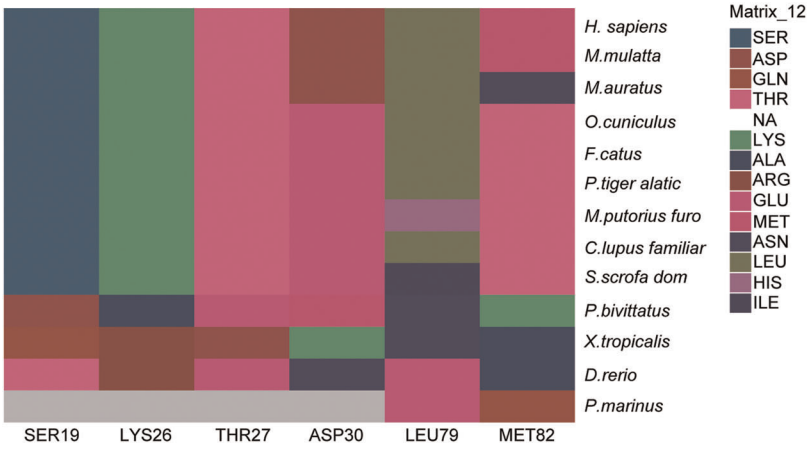
RBD, ACE2 from horse, cow, and malayan pangolin to RBD probably has less affinity of RBD to ACE2. Secondly, GOR IV prediction displays a similar composition of the secondary structure of ACE2 from different species, including alpha helix (average value is 41.7%), extended strand (average value is 17.53%), and random coil (average value is 40.78%), whereas the lower advanced species snake has a more extended strand than other higher species (Figure 3B).

Furthermore, the 3D structure of the full-length ACE2 from the other 7 animals was predicted by I-TASSER program. C-score and TM-score of the first-ranked model from 7 animals are from (−2, 0) and 0.49 ±0.15, respectively (data not shown). As scores range from (−5, 2) and a large value means more confidence in the model, our model, therefore, is a good model (Figure S3B–H). For TM-score, a value > 0.5 means the model has correct topology. To gain an understanding of the structural basis for the binding of RBD with ACE2, we focused on the key binding contact region with those 25 amino acids. The structural alignments by TM-align serve showed that the TM-score between human ACE2 (1R42A) and horse, cow, and Malayan pangolin is 0.99889, 0.99699, and 0.99867 respectively, while the human and the snake is 0.87201. Consistently, the 3D structure of the binding site region of human ACE-SARS-CoV-2 is almost completely overlapped with horse, cattle or Malayan pangolin ACE2 (Figure S3I–K), while partially overlapped with snake ACE2 (Figure S3L).

4 | DISCUSSION

To our acknowledge, according to the limited information available to date, several species including pet dogs, cats, lions, tigers, and farmed mink can be naturally infected with SARS-CoV-2 from humans and cause COVID-19. There is no evidence that the infected pet dogs and cats could spread SARS-CoV-2 to humans, thereby the risk of animals transmitting SARS-CoV-2 to humans is considered to be very low. However, the genetic code of SARS-CoV-2 detected in three infected people demonstrate great resemblance to the viral genetic code previously found in the mink at the infected farm in the Netherlands, suggesting SARS-CoV-2 is probably able to transmit

(A)



(B)

Species	Class/Order	Common Name	SER19	LYS26	THR27	ASP30	MET82	Probability of SARS-CoV-2 (+)
M.fascicularis	Primates/Eutheria	Crab-eating macaque	1	1	1	1	1	Y
M.musculus	Rodentia/Eutheria	Mouse	1	1	1	0	NA	NA
C.porcellus	Rodentia/Eutheria	Guinea pig	NA	1	1	1	0	NA
P.concolor	Carnivora/Eutheria	Cougar	1	1	1	1	1	Y
C.crocuta	Carnivora/Eutheria	Spotted hyena	1	1	1	1	1	Y
S.suricatta	Carnivora/Eutheria	Meerket	1	1	1	1	0	Y
P.larvata	Carnivora/Eutheria	Masked palm civet	1	1	1	1	1	Y
U.arctos horribilis	Carnivora/Eutheria	Grizzly bear	1	NA	1	1	1	NA
U.maritimus	Carnivora/Eutheria	Polar bear	1	NA	1	1	1	NA
A.melanoleuca	Carnivora/Eutheria	Panda	1	NA	1	1	1	NA
P.lotor	Carnivora/Eutheria	Raccoon	1	NA	1	1	1	NA
C.lupus dingo	Carnivora/Eutheria	Dingo (Dog)	1	1	1	1	1	Y
N.procyonoides	Carnivora/Eutheria	Raccoon dog	1	NA	1	1	1	NA
Vulpes vulpes	Carnivora/Eutheria	Red fox	1	NA	1	1	1	NA
M.javanica	Pholidota/Eutheria	Malayan pangolin	1	1	1	1	1	Y
O.aries	Artiodactyla/Eutheria	Sheep	1	1	1	1	1	Y
C.hircus	Artiodactyla/Eutheria	Goat	1	1	1	1	1	Y
B.taurus	Artiodactyla/Eutheria	Taurine cattle	1	1	1	1	1	Y
B.mutus	Artiodactyla/Eutheria	Wild Yak	1	1	1	1	1	Y
B.indicus	Artiodactyla/Eutheria	Humped cattle	1	1	1	1	1	Y
M.muntjak	Artiodactyla/Eutheria	Barking deer	1	1	1	1	1	Y
S.scrofa	Artiodactyla/Eutheria	Wide pig	1	1	1	1	1	Y
C.ferus	Artiodactyla/Eutheria	Camel	1	1	1	1	1	Y
E.caballus	Perissodactyla/Eutheria	Horse	1	1	1	1	1	Y
H.armiger	Chiroptera/Eutheria	Great Roundleaf bat	1	1	0	1	NA	NA
R.ferrumequinum	Chiroptera/Eutheria	greater horseshoe bat	1	1	NA	1	1	NA
T.chinensis	Scandentia/Eutheria	Chinese tree shrew	0	1	NA	0	NA	NA
D.novemcinctus	Edentata/Eutheria	Nine-banded armadillo	1	NA	1	1	1	NA
E.europaeus	Insectivora/Eutheria	Hedgehog	0	1	NA	1	1	NA
L.africana	Macroscelidea/Eutheria	Elephant	1	0	1	1	NA	NA
T.manatus latirostris	Tubulidentata/Eutheria	Sea Cow	1	0	1	1	1	Y
O.afer afer	Sirenia/Eutheria	Aardvark	NA	NA	1	1	NA	NA
E.edwardii	Proboscidea/Eutheria	Cape elephant shrew	NA	1	NA	1	1	NA
P.cinereus	Diprotodontia/Metatheria	Koala	NA	1	0	1	1	NA
O.anatinus	Monotremata/Prototheria	Platypus	NA	0	NA	NA	0	NA
G.gallus	Aves	Chicken	0	NA	1	NA	NA	NA
A.platyrhynchos	Aves	Duck	0	1	NA	NA	1	NA
A.forsteri	Aves	Penguin	0	NA	NA	1	NA	NA

FIGURE 2 (See caption on next page)

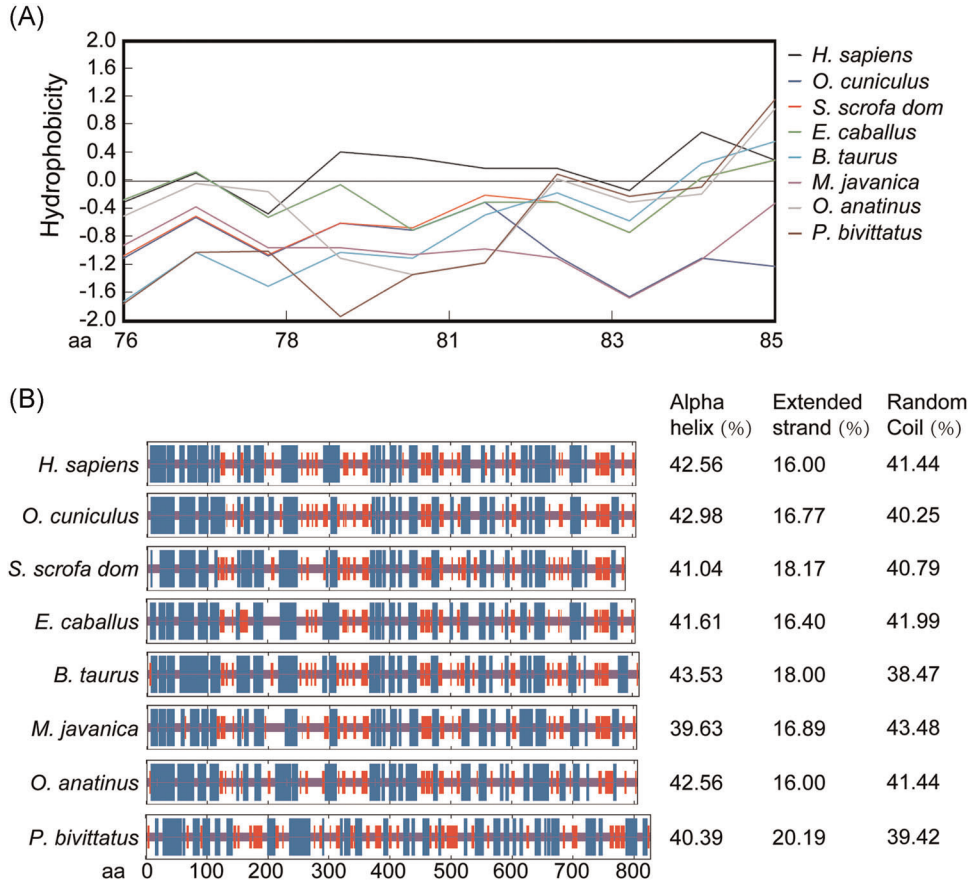


FIGURE 3 The hydrophobicity and secondary protein structure of ACE2 gene from representative animals. (A) The hydrophobicity of amino acid 76–85 of ACE2 genes was analyzed by DNAMAN with windows size 6 and scale from −2 to 2. (B) The secondary protein structure of ACE2 genes was analyzed by GOR IV prediction. The left is the overall architecture of the secondary protein structure and the right is the ratio of different elements. The blue rectangle indicates alpha-helix and the red rectangle indicates extended strand. ACE2, angiotensin-converting enzyme 2

from mink to human (the COVID-19 update 209 on International Society for infectious Disease). Particularly, some virus could end up passing back and forth between animals and people. For example, the 2009 pandemic H1N1 influenza virus originated in pigs, transmitted to humans, spread worldwide and then jumped back to pigs.[66,67] Notably, given the livestock and pets are closely connected to human beings and very few related investigations have been explored, more studies are needed to be conducted to understand if different animals could be affected by SARS-CoV-2, so as to avoid a potential outbreak by animal transmission in the further, which would become a critical aspect for controlling pneumonia.

4.1 | ACE2 and TMPRSS2 in SARS-CoV-2 cell entry

Coronavirus entry into host cells is an important determinant of viral pathogenesis. ACE2 and TMPRSS2 are known to function as a cell surface receptor for viral attachment and cell surface protease for viral-cell fusion, respectively.[36,37,68] To gain insight to viral cell entry, we first determined ACE2 and TMPRSS2 homologs from invertebrates to vertebrates and found both of two genes occur in vertebrates. Interestingly, compared to the stable existence and domain composition of ACE2 in each order of vertebrate, TMPRSS2

FIGURE 2 Probability of the interface of ACE2 from different species with SARS-CoV-2. (A) The heatmap of frequencies of human Ser19, Lys26, Thr27, Asp30, Leu79, and Met82 across different species. The heatmap was produced by Heatmap function from R library Complexheatmp. (B) Probability of the binding of ACE2 to SARS-CoV-2 from different species. A random forest analysis was generated by randomForest function from the R randomForest library. "1" means the amino acid is the same as those nine species which are known to be recognized by the virus, whereas "0" means is the same as those four species which do not have receptor binding. "NA" means "unknown"; "Y" means "Yes." ACE2, angiotensin-converting enzyme 2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

vary considerably across different species as following. First, TMPRSS2 is absent in many animals, even in some mammals; Secondly, the identities of TMPRSS2 between human and animals is much lower than the identities of ACE2 between human and animals; Thirdly, TMPRSS2 from ferret and great roundleaf bat lack the transmembrane region, indicating that TMPRSS2 on those two animals is not able to function as protease. Given the ferret and fruit bats is susceptible to SARS-CoV-2,[27] TMPRSS2 might be not necessary for viral cell entry in these animals, or some other redundant genes could replace the TMPRSS2 role. Coincidentally, a single-cell RNA-seq data analysis of *ACE2* and *TMPRSS2* from multiple tissues from healthy donors demonstrated that although no co-expression of ACE2 and TMPRSS2 in placenta/decidua and fetal liver and thymus, co-expression is observed in multiple tissue including airways, cornea, esophagus, ileum, colon, liver, gallbladder, heart, kidney and testis, in the eight subsets of $ACE2^+$ airway epithelial cells, including nasal, goblet, basal, and Type II alveolar, TMPRSS2 was only expressed in one subset of $ACE2^+$ cells, while cathepsin B, another protease is expressed in more than 70%–90% of $ACE2^+$ cells, indicating that cathepsin or other proteases probably functionally replaces TMPRSS2 during the cell entry of SARS-CoV-2, and along with that *ACE2*, rather than *TMPRSS2*, may be a limiting factor for viral entry.[69] Therefore, in this study, we focused on ACE2 on the following analysis, including sequence comparison, prediction of probability for infectious, and homology modeling and prediction.

4.2 | The N-glycosylation and hydrophobicity on the recognition of ACE2 to SARS-CoV-2

Receptor recognition is mainly determined by two factors, the binding specificity and affinity. A recent study identified the glycosylation at Asp90 could partly disrupt the interaction of the SARS-CoV-2 with ACE2 receptors,[58] whereas Wang et al.,[61] discovered the hydrophobic contacts at Leu79, Met82, and Tyr83 of ACE2 can enhance receptor recognition. Our predictions of the number and position of N-glycosylation sites appeared to vary across different species (Table 1). However, few studies about which N-glycosylation can play a role in receptor binding have been reported. It is therefore hard to calculate the correlation of glycosylation sites across species and their susceptibility to viral cell entry. To some extent, the various N-glycosylations might result in varied receptor affinity. Moreover, our prediction about hydrophobicity implied less receptor affinity in all animals than humans, consistent with the current phenomenon that the virus is now spreading from person to person.

4.3 | The prediction of SARS-CoV-2 transmissibility

The phylogenetic tree of ACE2 among different organisms imply that the ACE2 is highly conserved in mammals. In many different classes of mammals, there are organisms with confirmed receptor binding, indicating that other species from the same class are possibly susceptible to SARS-CoV-2 infections. Nevertheless, our analysis about the probability of viral attachment across different species in Figure 2B demonstrated the probability varies in the same class. Moreover, a recent study in the human community described the viral entry/receptor binding is not the only factor that determines viral infection in COVID-19 cases, while host age, underlying conditions, behavior, and population density can also determine infection.[70] It will be the same in the animals' world.

4.4 | SARS-CoV-2 transmissibility in pig

Chu et al.,[25] examined the greater increase of SARS-CoV-2 replicates in pig and rabbit cell lines, revealing that ACE2 can recognize SARS-CoV-2. Therefore we selected pig and rabbit ACE2 as the positive case for its binding to SARS-CoV-2 in the following analysis, including Figure 1–3 and Table 3. However, pig orally/intranasally/intratracheally inoculation inoculated with SARS-CoV-2 was negative for viral RNA in all organ samples and contact animals during all checkpoints within 21 days infections, declaring that the pig is not susceptible to SARS-CoV-2 in vivo.[27,57] The sharp contrast of studies between cell lines and in vivo raises a concern, the viral receptor binding predictions is not sufficient to determine the susceptibility to SARS-CoV-2 infection. In future, more experiments on viral transmission should be performed to study the susceptibility of animals to SARS-CoV-2.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

DATA AVALABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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