In silico analysis of intermediate hosts and susceptible animals of SARS-CoV-2

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Abstract

COVID-19, caused by SARS-CoV-2 with major symptom of pneumonia is bringing huge disasters to the people around the world. Recent research indicates that the natural host of SARS-CoV-2 may be bats, but its intermediate host is still unclear. Only by finding natural and intermediate host for SARS-CoV-2 can cut off the source and prevent the virus from being transmitted to humans. In this study, we established a new method for the mining of intermediate host. We selected 82 representative ACE2 sequences from the 1000 sequences with the closest homology to the human ACE2 protein. All these selected ACE2 proteins were modeled by homology modeling. The potential natural and intermediate hosts, as well as susceptible animals of SARS-CoV-2 were analyzed systematically by calculating the binding free energy of ACE2 protein with the RBD of SARS-CoV-2. Based on this study, *Rhinolophus sinicus* was suggested to be the natural host, and the virus may be transmitted directly from bats to humans. Primates, some wild Felidae, civet, goats, spotted hyenas and golden hamsters may be susceptible to SARS-CoV-2 and could be intermediate hosts, while pangolins are unlikely to be intermediate hosts, and birds and reptiles are not intermediate hosts. Mice, rats and guinea pig are not susceptible to SARS-CoV-2. Considering the possible susceptibility of non-human primates, goats and golden hamsters, they can be used as experimental animals directly for the SARS-CoV-2 infection models without transgenic operation. Herein, the possible candidates of natural and intermediate hosts of SARS-CoV-2 were suggested, which will provide guiding significance for subsequent researches.

1. Introduction

The world is going through a smokeless war against the novel coronavirus (SARS-CoV-2) in 2020. By the end of March 2020, there were more than 710,000 COVID-19 patients and 30,105 deaths worldwide. The epidemic has affected 202 countries and regions around the world, and the WHO says it will take 12 months for vaccine development. Inevitably, this is the obstacle that all humanity needs to work together to overcome it. Humans have experienced three outbreaks of pneumonia

due to coronavirus since entering the 21st century, which strongly reminds us that we must pay sufficient attention to coronavirus, and its prevention and treatment. Tracing the origin of the SARS-CoV-2 and its route of transmission is of great significance for the development of treatment and prevention for future re-epidemic.

According to the transmission route of the virus, the host of the virus is generally divided into a natural host, an intermediate host and a final host. Intermediate hosts of a virus may include multiple species as a vehicle to "transport" the virus from the natural host to the final host. In order to control the further spread of the virus, besides quarantining and treating already infected patients, the discovery and isolation of intermediate hosts can actually block the infection from the source. Palm civets were confirmed to be the main intermediate host for SARS-CoV [1] and dromedary camels for MERS-CoV [2], all of which were proven to originate from bats [3-5]. Shi ZL, *et al* found that the sequence identity of SARS-CoV-2 and the bat coronavirus RaTG13 (Bat-CoV-RaTG13) carried in *Rhinolophus affinis* from Yunnan Province of China was 96.2%. Furthermore, the sequence identity of *S* gene (encoding the spike protein) of SARS-CoV-2 and Bat-CoV-RaTG13 was 93.1%, which is much higher than other SARS-CoVs [6]. It is believed that the natural host of SARS-CoV-2 is also bats.

Currently, research on intermediate hosts for SARS-CoV-2 is underway, and the research objects include pangolins, minks, turtles, *etc*. In four reported studies, the genome sequence similarity between pangolin-CoVs and SARS-CoV-2 was 85.5% to 92.4% [7], 91.02% [8], 90.3% [9], and 90.23% [10], respectively. There are two species of SARS-CoV-2 related pangolin-CoVs, pangolin-CoV GD and pangolin-CoV GX, which meant the original source of pangolins was found in Guangdong and Guangxi Province of China respectively. The researchers found that although SARS-CoV-2 is closest to Bat-CoV-RaTG13 in other regions, SARS-CoV-2 has a high sequence similarity with the receptor binding domain of pangolin-associated coronaviruses. One of studies showed that pangolin-CoV GD exhibited strong similarity to SARS-CoV-2 in the receptor-binding domain, 97.4% identity in amino acid sequence, which is

better than Bat-CoV-RaTG13 (89.2%)[7]. There are also three studies supporting this result, showing that the RBD of pangolin-CoV GD and SARS-CoV-2 is highly conserved with only one amino acid residue difference [8-10]. Furthermore, pangolin-CoVs and SARS-CoV-2 have the same amino acids on five key residues of RBD, but Bat-CoV-RaTG13 has only one amino acid residue consistent with SARS-CoV-2 [7, 8]. Researchers also suggested that the amino acid similarity between pangolin-associated coronaviruses RBD and SARS-CoV-2 may be due to selective mediated convergence evolution rather than recombination.

However, the SARS-CoV-2 spike protein has a special "PRRA" motif insertion at the S1/S2 cleavage site [7, 8, 10, 11] and this motif is not found in Bat-CoV-RaTG13 or pangolin-CoVs. Chen J, *et al* thought that this motif may be inserted by other intermediate hosts during viral transmission [10]. Therefore, whether pangolins are intermediate hosts of SARS-CoV-2 still needs a large amount of experimental samples and data analysis. Zhu H, *et al* found that mink coronavirus showed an infection pattern closer to SARS-CoV-2 according to deep learning algorithms, suggesting that minks may be an intermediate host for SARS-CoV-2 [12]. Meanwhile, a study suggests that turtles may be intermediate hosts for SARS-CoV-2 [13].

At present, the intermediate host of SARS-CoV-2 has not been determined, and most researchers believe that there are more than one intermediate hosts. Other researchers think that intermediate hosts may not be needed and the virus can directly infect human. Most of the researches only involved the identity analysis of genomic sequence between the potential intermediate host and SARS-CoV-2, and the similarity analysis of some protein domains. No research team is currently conducting experimental verification.

Here, we selected angiotensin-converting enzyme 2 (ACE2) sequences from other species with the closest homology to human ACE2 protein, including Primates, Chiroptera, Felidae, Canidae, Circetidae, Camelidae, and previously reported *Manis javanica*, *Mustela putorius furo*, *etc*. These species were divided into different families by sequence alignment and phylogenetic tree analysis, and homology

modeling of all ACE2 proteins. Protein-protein docking of SARS-CoV-2 spike with ACE2 of different species and calculation of binding free energy were performed to find potential intermediate hosts or susceptible animals for SARS-CoV-2. In addition, two coronavirus spike with the highest similarity to SARS-CoV-2 spike were modeled, docked with human ACE2 and various ACE2 to calculate the free energy in order to determine the possibility of these coronavirus directly infecting humans and other animals. In a word, we set up a new approach for the mining of intermediate hosts, systematically analyze the potential natural and intermediate hosts of SARS-CoV-2 by calculating the binding free energy between RBD and ACE2, and also provide suggestions for the selection of experimental animals for COVID-19.

2. Method

2.1 Homology ACE2 protein blast and sequence alignment.

Amino acid sequence editing was conducted using Bioedit and DNAMAN, and sequence alignment was conducted using Clustalw. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 7 software package. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was determined by 1000 replicates. 3D structure structures were analysised by pymol tool.

The full length ACE2 sequence (NP_001358344.1) was downloaded from NCBI protein database. The amino acid sequence were aligned with whole database using BLASTp to search for homology ACE2 protein (Alogorithm parameters, max target sequences: 1000, expect threshold: 10). Accession numbers of 82 chosen ACE2 sequences are listed as follows: Gorilla gorilla gorilla (XP_018874749.1), Macaca nemestrina (XP_011733505.1), Aotus nancymaae (XP_012290105.1), Ictidomys tridecemlineatus (XP_005316051.3), Chinchilla lanigera (XP_013362428.1), Oryctolagus cuniculus (XP_002719891.1), Urocitellus parryii (XP_026252505.1), Marmota marmota marmota (XP_015343540.1), Fukomys damarensis (XP_010643477.1), Marmota flaviventris (XP_027802308.1), Heterocephalus glaber (XP_004866157.1), Equus przewalskii (XP_008542995.1), Felis catus

(NP_001034545.1), Camelus ferus (XP_006194263.1), arlito syrichta(XP_008062810.1), Manis javanica (XP_017505746.1), Crocuta crocuta(KAF0878287.1), Capra hircus (NP_001277036.1), Ovis aries (XP_011961657.1), Sus scrofa (NP_001116542.1), Mustela putorius furo (NP_001297119.1), Canis lupus dingo (XP_025292925.1), Camelus dromedarius (KAB1253106.1), Vulpes vulpes (XP_025842512.1), Tupaia chinensis (XP_006164754.1), Canis lupus familiaris (NP_001158732.1), Sus scrofa domesticus (ACT66265.1), Orycteropus afer afer (XP_007951028.1), Puma concolor (XP_025790417.1), Ursus maritimus (XP_008694637.1), Panthera pardus (XP_019273508.1), Microtus ochrogaster (XP_005358818.1), Ursus arctos horribilis (XP_026333865.1), Lynx pardinus (VFV30336.1), Octodon degus (XP_023575315.1), Panthera tigris altaica(XP_007090142.1), Ceratotherium simum simum (XP_004435206.1), Ailuropoda melanoleuca (XP_002930657.1), Vicugna pacos (XP_006212709.1), Jaculus jaculus (XP_004671523.1), Balaenoptera acutorostrata scammoni (XP_028020351.1), Mesocricetus auratus (XP_005074266.1), Nyctereutes procyonoides (ABW16956.1), Phodopus campbelli (ACT66274.1), Equus asinus (XP_014713133.1), Dasypus novemcinctus (XP_004449124.1), Grammomys surdaster (XP_028617961.1), Mastomys coucha (XP_031226742.1), Loxodonta africana (XP_023410960.1), Meleagris gallopavo (XP_019467554.1), Phasianus colchicus (XP_031451919.1), Struthio camelus australism (XP_009667495.1), Crocodylus porosus (XP_019384827.1), Cavia porcellus (ACT66270.1), Phascolarctos cinereus(XP_020863153.1), Rhinolophus macrotis (ADN93471.1), Rhinolophus pearsonii (ABU54053.1), Ophiophagus hannah (ETE61880.1), Paguma larvata (Q56NL1.1), Mus musculus (NP_001123985.1), Rattus norvegicus (NP_001012006.1), Macaca fascicularis (XP_005593094.1), Macaca mulatta (ACI04556.1), Papio anubis (XP_021788732.1), Erinaceus europaeus (XP_007538670.1), Bos mutus (XP_005903173.1), Rhinolophus sinicus (ADN93475.1), Rhinolophus landeri (ALJ94034.1), Rhinolophus alcyone (ALJ94035.1), Rhinolophus ferrumequinum (ADN93470.1), Rhinolophus pusillus (ADN93477.1), Pteropus alecto

(XP_006911709.1), Rousettus aegyptiacus (XP_015974412.1), Rousettus leschenaultii (ADJ19219.1), Myotis lucifugus (XP_023609437.1), Pteropus vampyrus (XP_011361275.1), Eptesicus fuscus (XP_008153150.1), Miniopterus natalensis (XP_016058453.1), Myotis davidii (XP_006775273.1) Myotis brandtii (XP_014399782.1), Pipistrellus abramus (ACT66266.1). Spike protein sequences used in this study: SARS-CoV-2 (YP_009724390.1), SARS-CoV (AAS00003.1) bat-CoV-RaTG13 (QHR63300.2), Pangolin-CoV/GD (GD EPI_ISL_410544), Pangolin-CoV/GD (GX EPI_ISL_410538)

2.2. Homology modeling and molecular docking

Base on the recent disclosed structure of SARS-CoV-2 Spike RBD-ACE2 complex (PDB code: 6LZG) [14], corresponding homology models of each spike RBD and ACE2 were built. Alignment of two protein sequences and subsequent homology modeling were performed by bioinformatics module of ICM 3.7.3 modeling software on an Intel i7 4960 processor (MolSoft LLC, San Diego, CA) [15]. Protein-protein docking procedure was performed according to the ICM-Pro manual, and the free binding energy was calculated.

3. Results

3.1 Bioinformatics analysis of ACE2 proteins

1000 homology sequences of human ACE2 protein were found through BLASTp method. ACE2 sequences from 82 species were chosen and performed a phylogenetic tree analysis (**Figure 1**). 82 species mainly belong to *mammalia*, and a few from other class, such as *Aves*, *Reptilia*, and *Sauropsida*. Among those mammals, the mammalian group includes primates, rodents, odd hoofs, artiodactyls, carnivores, lagomorphs, bats, and so on. Bats are proposed to be the natural host of SARS-CoV-2 [16]. In order to find possible source hosts, we collected all available ACE2 sequences from *Chiroptera*, with total number of 17.

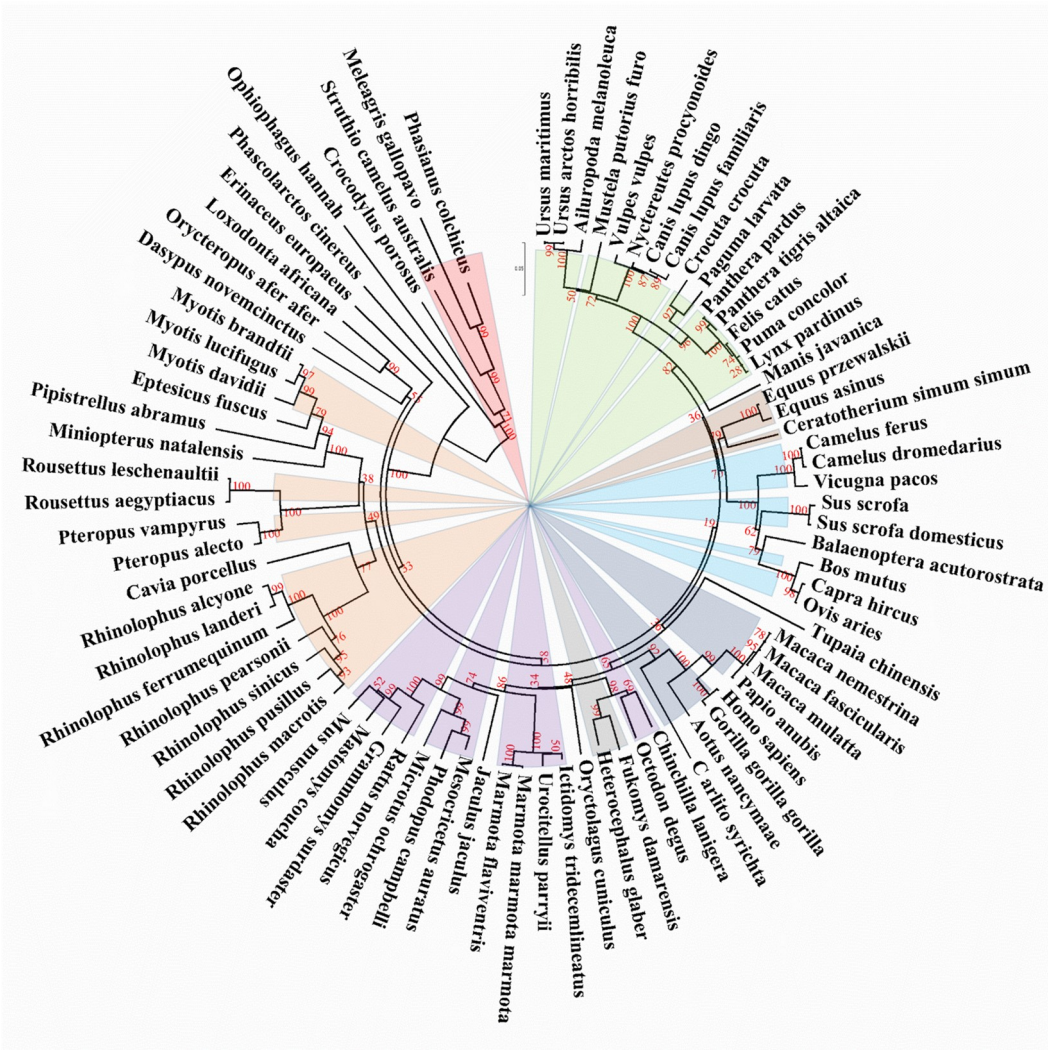

Figure 1 Sequence phylogeny of the complete ACE2 proteins from 82 species.

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 3.96647534 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 82 ACE2 amino acid sequences from different species. All positions containing gaps and missing data were eliminated. There were a total of 625 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. Species included in the same circular sector are from the same family. Species from the same order is marked with circular sector in the same color. Those that were not marked species came from separate orders.

At present, the structure of the hACE2 and SARS-CoV-2 Spike-RBD complex has been resolved [14, 17], as shown in **Figure 2 A** and **2B**. The seven amino acids at the hACE2 binding interface and Spike-RBD form eight hydrogen bonding interactions, including Gln24, Asp30, His34, Tyr41, and Gln42 in hACE2 form hydrogen bonding interactions with Gln474, Lys417, Tyr453, Asn501, and Gln498 in SARS-CoV-2 spike-RBD, respectively, among them, two hydrogen bonds were formed between Q42 in

hACE2 and Gln498 in spike-RBD. What's more, Lys353 and Arg357 in hACE2 are respectively interacts with Asn501 and Thr500 in spike with hydrogen bonds (**Figure. 2A** and **2B**). In addition, Met82 in ACE2 interacts with Phe486 in spike-RBD through Van der Waals force. We also analyzed the binding pattern of ACE2 from *Rhinolophus sinicus* and *Mesocricetus auratus* with Spike-RBD from SARS-CoV-2 through docking model. Both of them can also form 8 hydrogen bonds. From the results of sequence comparison, it is seen that there are two key amino acids in *Rhinolophus sinicus* ACE2 sequence that differ from human (**Figure 2G)**. In *Rhinolophus sinicus*, ACE2 sequence is Arg24 instead of Gln24 ，and Ser34 instead of His34. Arg24 and Ser34 interact with Ser 477and Q493 with hydrogen bonds, respectively (**Figure 2C, 2D, 2G and 2H**). There is only one key amino acid in *Mesocricetus auratus* ACE2 sequence that differs from human (**Figure 2E-H**). In *Mesocricetus auratus*, ACE2 sequence is Gln34 rather than His34, but Gln34 can also form a hydrogen bonding interaction with Tyr453. However Gln24 in *Mesocricetus auratus* ACE2 forms a hydrogen bonding interaction with Asn487 instead of Gln474. The key amino acids ACE2 and Spike-RBD interactions are marked in **Figure 2G** and **2H**.

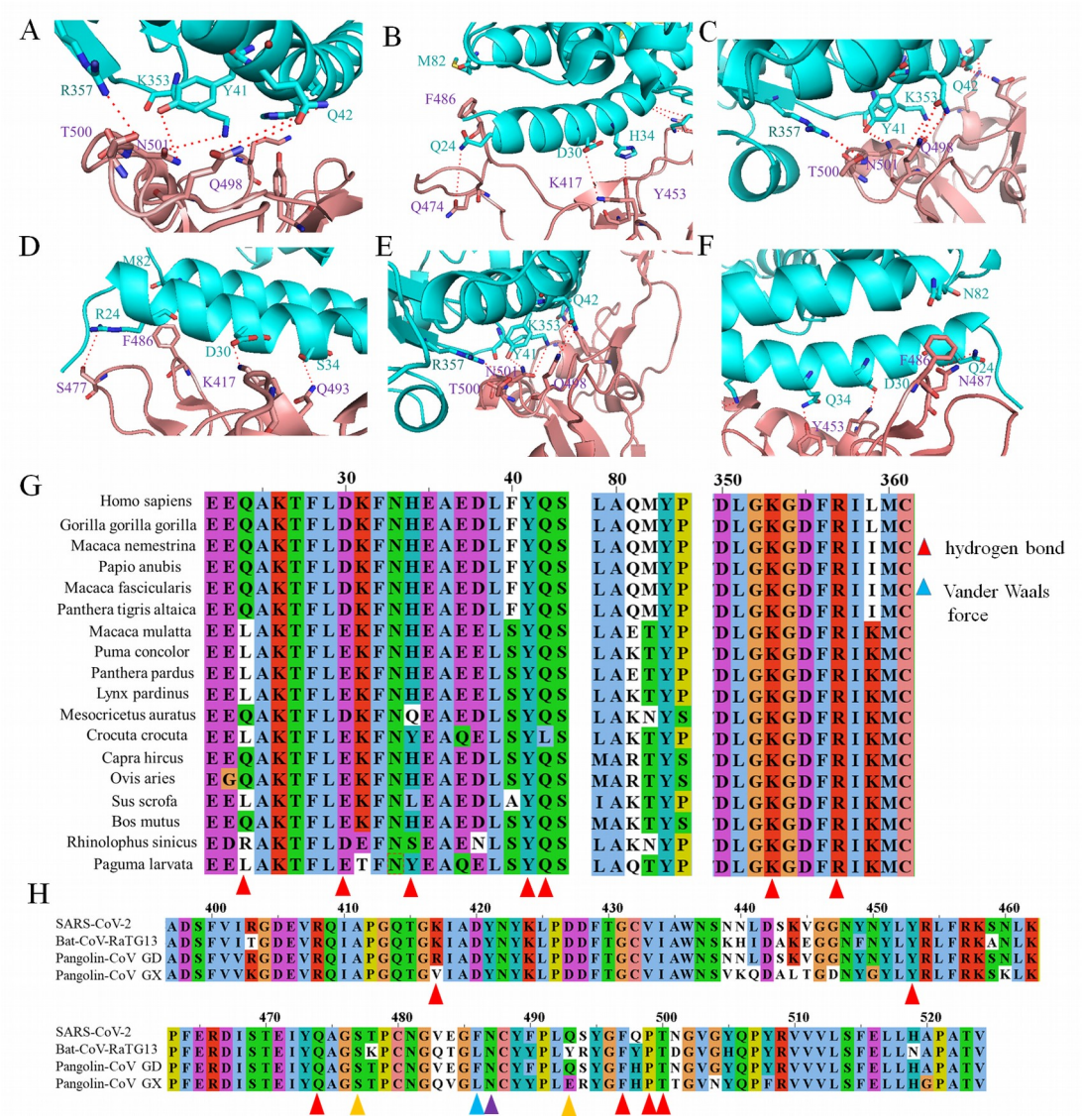

Figure 2. Analysis of key amino acids at the interface of ACE2 and SPIKE-RBD.

A and **B**, hACE2 and SARS-CoV-2 Spike-RBD interaction interface analysis. These pictures were plotted with pymol using ACE2-Spike-RBD complex as a model (PDB code: 6m17). hACE2 was displayed as cartoon mode in blue. SARS-CoV-2 displayed as cartoon mode in brown, and these binding amino acids were displayed in stick mode. Those red sticks represent oxygen atoms, those blue sticks represent nitrogen atoms, and those red dotted lines represent hydrogen bond. **C** and **D**. Analysis of the interaction interface between *Rhinolophus sinicus* ACE2 and SARS-CoV-2 Spike-RBD. **E** and **F**. Analysis of the interaction interface between *Mesocricetus auratus* ACE2 and SARS-CoV-2 Spike-RBD. **G**. ACE2 from 18 species which docked with SARS-CoV-2 spike-RBD with binding free energy below -49Kj/mol. sequence alignment of ACE2 spike binding motif, in which amino acids bound to Spike-RBD with hydrogen bonds were marked with red triangles below, and amino acids binding to RBD with van der Waals force were marked with blue triangle below. **H.** Sequence alignment of four coronavirus receptor binding motifs. Amino acids bound to human ACE2 with hydrogen bonds were marked with red triangles below. Amino acids that predicted binding to *Rhinolophus sinicus* ACE2 with hydrogen bonds were marked with yellow triangles below. Amino acids that predicted binding to *Mesocricetus*

auratus ACE2 with hydrogen bonds were marked with purple triangles below.

3.2 Homology modeling and protein-protein docking calculation

All ACE2 protein structures were homology modeled by ICM modeling software using the human ACE2 structure as the template. Then the binding free energy was calculated by docking the sipke protein of SARS-CoV-2 or other coronaviruses with each ACE2 protein. In most of case, the generated conformation resembling the crystal structure of human ACE2-SARS-CoV-2 RBD complex was the conformation with the minimum energy. The results obtained are shown in **Table 1** and **Table 2**.

Table 1 Binding free energy of human ACE2 with Spike RBD from different coronavirus calculated by protein-protein docking

NO.	Virus Name	RBD Similarity to SARS-CoV-2	Free binding energy ($KJ.mol^{-1}$)
1	SARS-CoV-2	100%	-50.1326
2	SARS-CoV	74.6%	-49.2229
3	Bat RaTG13	89.2%	-44.9803
4	Pangolin-CoV GD	97.1%	-48.0341
5	Pangolin-CoV GX	87.1%	-40.1424

As shown in Table 1, among all five closely related SARS family coronavirus, SARS-CoV-2 spike RBD seems to have the strongest affinity to human ACE2, which is consistent with the observation of high infectivity of SARS-CoV-2. Although SARS-CoV RBD shares the lowest similarity with that of SARS-CoV-2, its calculated binding affinity was closest to SARS-CoV-2, and now we know they have slightly different binding mode in the interface as shown in the complex structures [14, 17]. For other three SARS-CoV-2 closely related coronavirus, the closer the similarity of its RBD is, the lower the free binding energy it has. We further chose SARS-CoV-2 and two most similar viruses, Bat RaTG13 and Pangolin-CoV GD for protein-protein docking studies.

Table 2 Binding free energy of SARS-CoV-2 RBD with ACE2 from different species

calculated by protein-protein docking

NO.	Species Name	Similarity	Accession Number	Free binding energy ($KJ.mol^{-1}$)
1	*Homo sapiens*	100%	NP_001358344.1	-50.1326
2	*Gorilla gorilla*	99.01%	XP_018874749.1	-51.5556
3	*Macaca nemestrina*	95.34%	XP_011733505.1	-51.5325
4	*Papio anubis*	95.34%	XP_021788732.1	-51.5628
5	*Macaca fascicularis*	95.21%	XP_005593094.1	-51.5373
6	*Macaca mulatta*	95.21%	ACI04556.1	-51.5677
7	*Aotus nancymaae*	92.17%	XP_012290105.1	-42.8772
8	*Equus przewalskii*	86.90%	XP_008542995.1	-48.8959
9	*Ceratotherium simum*	85.77%	XP_004435206.1	-48.3243
10	*Panthera tigris*	85.70%	XP_007090142.1	-50.6125
11	*Puma concolor*	85.59%	XP_025790417.1	-50.5544
12	*Panthera pardus*	85.47%	XP_019273508.1	-50.6849
13	*Ictidomys tridecemlineatus*	85.38%	XP_005316051.3	-48.8769
14	*Felis catus*	85.22%	NP_001034545.1	-48.8741
15	*Lynx pardinus*	85.22%	VFV30336.1	-50.6549
16	*Oryctolagus cuniculus*	85.14%	XP_002719891.1	-48.5832
17	*Marmota marmota*	84.88%	XP_015343540.1	-48.6519
18	*Urocitellus parryii*	84.76%	XP_026252505.1	-47.6377
19	*Marmota flaviventris*	84.76%	XP_027802308.1	-48.6645
20	*Manis javanica*	84.76%	XP_017505746.1	-46.3551
21	*Chinchilla lanigera*	84.72%	XP_013362428.1	-43.1693
22	*Fukomys damarensis*	84.72%	XP_010643477.1	-42.1498
23	*Jaculus jaculus*	84.63%	XP_004671523.1	-46.0314
24	*Heterocephalus glaber*	84.60%	XP_004866157.1	-42.0874
25	*Octodon degus*	84.47%	XP_023575315.1	-35.7756
26	*Mesocricetus auratus*	84.26%	XP_005074266.1	-50.4353
27	*Arlito syrichta*	84.10%	XP_008062810.1	-37.8413
28	*Canis lupus dingo*	84.01%	XP_025292925.1	-40.7918
29	*Nyctereutes procyonoides*	84.01%	ABW16956.1	-43.609
30	*Ursus maritimus*	83.92%	XP_008694637.1	-45.0617
31	*Ursus arctos*	83.88%	XP_026333865.1	-45.0899
32	*Vulpes vulpes*	83.63%	XP_025842512.1	-45.4803
33	*Microtus ochrogaster*	83.63%	XP_005358818.1	-44.1707
34	*Canis lupus familiaris*	83.50%	NP_001158732.1	-40.7225
35	*Paguma larvata*	83.48%	Q56NL1.1	-49.3514
36	*Equus asinus*	83.40%	XP_014713133.1	-48.0456
37	*Ailuropoda melanoleuca*	83.38%	XP_002930657.1	-45.2657
38	*Crocuta crocuta*	83.35%	KAF0878287.1	-50.1934
39	*Vicugna pacos*	83.35%	XP_006212709.1	-44.6744
40	*Camelus ferus*	83.23%	XP_006194263.1	-47.3657
41	*Phodopus campbelli*	82.87%	ACT66274.1	-44.875

42	*Mustela putorius*	82.74%	NP_001297119.1	-45.3724
43	*Balaenoptera acutorostrata*	82.48%	XP_028020351.1	-42.7212
44	*Rattus norvegicus*	82.37%	NP_001012006.1	-47.2193
45	*Grammomys surdaster*	82.24%	XP_028617961.1	-46.6804
46	*Sus scrofa domesticus*	81.94%	ACT66265.1	-48.9879
47	*Mus musculus*	81.86%	NP_001123985.1	-44.6578
48	*Capra hircus*	81.74%	NP_001277036.1	-49.5148
49	*Pteropus alecto*	81.49%	XP_006911709.1	-47.2126
50	*Ovis aries*	81.74%	XP_011961657.1	-49.6762
51	*Mastomys coucha*	81.38%	XP_031226742.1	-46.7412
52	*Sus scrofa*	81.37%	NP_001116542.1	-49.0061
53	*Rhinolophus pearsonii*	81.37%	ABU54053.1	-46.2924
54	*Bos mutus*	81.37%	XP_005903173.1	-49.4998
55	*Camelus dromedarius*	80.87%	KAB1253106.1	-47.28
56	*Rhinolophus macrotis*	80.87%	ADN93471.1	-48.9215
57	*Tupaia chinensis*	80.75%	XP_006164754.1	-39.509
58	*Miniopterus natalensis*	80.75%	XP_016058453.1	-43.4486
59	*Rhinolophus sinicus*	80.62%	ADN93475.1	-50.4141
60	*Rhinolophus landeri*	80.62%	ALJ94034.1	-46.5592
61	*Pteropus vampyrus*	80.62%	XP_011361275.1	-46.333
62	*Loxodonta africana*	80.50%	XP_023410960.1	-45.8706
63	*Rhinolophus alcyone*	80.50%	ALJ94035.1	-46.4305
64	*Rhinolophus ferrumequinum*	80.50%	ADN93470.1	-46.4919
65	*Eptesicus fuscus*	80.42%	XP_008153150.1	-35.0887
66	*Myotis brandtii*	80.37%	XP_014399782.1	-46.1067
67	*Rhinolophus pusillus*	80.35%	ADN93477.1	-48.041
68	*Myotis lucifugus*	80.25%	XP_023609437.1	-44.8588
69	*Cavia porcellus*	79.54%	ACT66270.1	-37.9728
70	*Orycteropus afer*	79.38%	XP_007951028.1	-46.2635
71	*Myotis davidii*	79.15%	XP_006775273.1	-46.8656
72	*Rousettus leschenaultii*	79.13%	ADJ19219.1	-44.8589
73	*Dasypus novemcinctus*	79.13%	XP_004449124.1	-40.5196
74	*Erinaceus europaeus*	79.01%	XP_007538670.1	-49.2088
75	*Rousettus aegyptiacus*	78.88%	XP_015974412.1	-35.4247
76	*Pipistrellus abramus*	76.45%	ACT66266.1	-40.3802
77	*Phascolarctos cinereus*	71.48%	XP_020863153.1	-36.0763
78	*Crocodylus porosus*	67.45%	XP_019384827.1	-40.4653
79	*Phasianus colchicus*	66.09%	XP_031451919.1	-36.1372
80	*Struthio camelus*	65.01%	XP_009667495.1	-45.8706
81	*Ophiophagus hannah*	56.91%	ETE61880.1	-34.6833
82	*Meleagris gallopavo*	55.50%	XP_019467554.1	-37.6367

From the results in **Table 2**, it can be seen that the binding force of ACE2

receptors and SARS-CoV-2 RBD in various animals basically follows the rule that the lower the homology with human, the weaker the binding force was observed, but there are some exceptions.

As shown in **Table 2**, ACE2 from primates (*Macaca mulatta*, *Papio anubis*, *Gorilla gorilla*, *Macaca fascicularis*, *Macaca nemestrina*) have stronger binding with the RBD of SARS-CoV-2 than that of homo sapiens (-50.1326 KJ.mol^{-1}), with lower free binding energy than -51 KJ.mol^{-1}. In primates, because ACE2s are highly homologous to human ACE2, they have strong binding force to RBD and even higher than human ACE2. Among them there is an exception, *Aotus nancymaae* ACE2 shows 92.17% homology with human ACE2, but the binding ability with RBD is significantly lower than that of human ACE2 and even lower than some birds.

For most of Felidae selected in this study, their ACE2 have stronger binding with the RBD of SARS-CoV-2 than that of homo sapiens, like *Panthera pardus*, *Lynx pardinus*, *Panthera tigris* and *Puma concolor*, with lower free binding energy than -50.1326 KJ.mol^{-1}. However, ACE2 of domestic cat has a little higher free binding energy than that of human, with the number of -48.8741 KJ.mol^{-1}. Worth to mention, for Canidae, including domestic dog, their ACE2 have much higher free binding energy than that of human, this means much weaker binding.

However, species that are more distantly related to humans, including *Mesocricetus auratus* and *Crocuta crocuta*, ACE2 receptors and RBD of SARS-CoV-2 have stronger binding than Homo sapiens. As shown in **Table 2**, the homology of rodentia ACE2s and human ACE2 is basically 81%-86%, for rats and mice, their binding ability to RBD is significantly weaker than that of human ACE2, but golden hamster has higher binding ability compared to humans.

Paguma larvata were confirmed to be the main intermediate host for SARS-CoV [1]. Our prediction results show that *Paguma larvata* and *Erinaceus europaeus* have similar binding ability with humans, indicating that these two species are also susceptible to SARS-CoV-2. *Erinaceus europaeus* ACE2 has only 79.01% homology with human ACE2, but its binding ability to RBD is very close to that of human.

Rhinolophus pearsonii and *Rhinolophus macrotis* belonging to Rhinolophidae,

share 81.37% and 80.87% homology with human ACE2, but they have relatively close binding ability to RBD compared with human. *Rhinolophus sinicus* ACE2 shares 80.62% homology with that of humans, but its binding ability to RBD is even stronger than that of humans. This suggests that bats may still be natural hosts for SARS-CoV-2.

Recent findings suggest that SARS-CoV-2 has most similar genetic information with bat coronovirus and most similar codon usage bias with snake [14]. However, there is much controversy about this conclusion. So we focused on the possibility of non-mammals as intermediate hosts. As shown in **Table 2**, the homology of non-mammalian (Phasianidae, Struthionidae, Elapidae, Phasianidae) ACE2 and human ACE2 is only 55%-66%, and they all have weak RBD binding ability to SARS-CoV-2. These results indicate that non-mammals (reptiles and birds) cannot be the intermediate hosts for SRAS-CoV-2.

In order to better compare the natural host and intermediate host of bat coronavirus RaTG13 and human SARS-CoV-2 virus, we docked the sipke RBD of bat coronavirus RaTG13 with ACE2 protein from different sources and calculated the binding free energy as well (Supplementary Table 1). The binding forces of RaTG13 RBD with ACE2 of various animals are basically similar to that of human SARS-CoV-2 virus in trend (**Supplementary Table 1**). The results show that the homology of *Capra hircus* ACE2 with that of human is 81.74%, and its binding ability to RaTG13 RBD is even stronger than that of human. In addition, ACE2 of *Rhinolophus macrotis* shares 80.87% homology with human ACE2, but its binding ability to RaTG13 RBD is comparable to human. Therefore, *Rhinolophus macrotis* may be the natural host of RaTG13. In addition, *Mesocricetus auratus*, *Jaculus jaculus*, *Ovis aries*, *Heterocephalus glaber*, and *Phodopus campbelli* also have strong binding force with the RBD of RaTG13.

Further analysis of the binding ability of ACE2 of various animals with the RBD of pangolin-CoV GD was performed (**Supplementary Table 2**), and we found that *Capra hircus*, *Homo sapiens*, *Mesocricetus auratus*, and *Marmota marmota* have strong

binding ability to the RBD of pangolin-CoV GD, especially *Capra hircus*. These species may be better intermediate hosts than *Manis javanica*.

Conclusion

The COVID-19 epidemic caused by novel coronavirus (SARS-CoV-2) has spread around the world. It is well known that viruses cannot grow and replicate independently, and can only replicate themselves in the host's living cells. Previously, researchers have suggested that bats may be natural hosts for SARS-CoV-2, and snakes, pangolins, turtles, and minks may be potential intermediate hosts [7-9, 12-13]. It is important to find the "intermediate host" of SARS-CoV-2. Only by finding it can cut off the source and prevent the virus from being transmitted to humans. However, to confirm the "intermediate host", the rigorous and recognized scientific process is as follows: (1) a virus that can reproduce continuously in the "intermediate host" is isolated; (2) the isolated virus can be displayed on animal models with disease and pathological characteristics; (3) confirm the position of the virus in the infection transmission chain, and so on. At present, the problem of intermediate host of SARS-CoV-2 is inconclusive. Some people have suggested that the virus may not need an intermediate host and directly transmit to humans from a natural host.

In this study, we selected 82 representative ACE2 sequences from the 1000 sequences that have the closest homology to the human ACE2 protein. Most of these species are mammals, and some of them are birds and reptiles. Through sequence alignment and phylogenetic tree analysis, these species were divided into different families, and the ACE2 protein of all species was modeled by homology. The Spike RBD of SARS-CoV-2 was docked with different ACE2 proteins, and the binding free energy was calculated. Results show the lower the homology between the species and human, the weaker the binding ability of its ACE2 receptor to the RBD domain of SARS-CoV-2.

Previous researches have suggested that the natural host of SARS-CoV-2 may be bats [6, 7], but its exact host remains unknown. From the analysis of our results, we found that *Rhinolophus sinicus* ACE2 has a little stronger binding ability to SARS-CoV-

2 RBD than that of humans. This suggests that *Rhinolophus sinicus* could be the natural host for SARS-CoV-2. And also considering the closed binding affinity of human and *Rhinolophus sinicus* ACE2 against spike RBD, it is possible that SARS-CoV-2 could be transmitted directly from bats to human being.

Our results show that most of primates, *Crocuta crocuta*, *Mesocricetus auratus*, and wild felines have stronger binding to the RBD domain of SARS-CoV-2 than that of humans, this implies that these animals may be intermediate hosts for SARS-CoV-2. For most of primates, including *Gorilla gorilla*, *Macaca nemestrina*, *Macaca fascicularis*, *Macaca mulatta* (Rhesus macaques) and *Papio anubi*s are suggested to be susceptible to SARS-CoV-2, this prediction was consistent with the previous study that conjunctival infection of SARS-CoV-2 can cause mild covid-19 in rhesus monkeys [19]. Based on this study, most of wild felines are susceptible to SARS-CoV-2. However, considering the relatively less possible touch between wild felines and humans, they are actually unlikely to be intermediate hosts. The domestic cat (*Felis catus*) ACE2 has weaker binding than that of wild felines, but considering its free energy is still close to that of human, the cats may still be a slightly susceptible to SARS-CoV-2. In contrast, dogs seem to be much less susceptible. These results are consistent with very recent results in bioXiv [20].

For animals like *Paguma larvata*, *Erinaceus europaeus*, *Erinaceus europaeus*, *Bos mutus*, *Ovis aries*, *Capra hircus and Sus scrofa*, their ACE2 all have slightly higher binding energy to SARS-CoV-2 RBD than that of human. Since the values are very close, we can speculate that these animals are susceptible to SARS-CoV-2, and they all could be the intermediate hosts.

Recent study showed that the RBD of pangolin-CoV GD and SARS-CoV-2 is highly conserved with only one amino acid residue difference, therefore suggesting that *Manis javanica* is the intermediate host of SARS-CoV-2 [7, 9]. However, our docking results show that the ACE2 receptor of pangolins does not bind strongly to the RBD of SARS-CoV-2, and this indicated that pangolins are not intermediate hosts of SARS-CoV-2. Furthermore, analysis of the binding ability of ACE2 of various

animals with the RBD of pangolin-CoV GD revealed that ACE2 of human and many animals other than *Manis javanica*, like *Capra hircus*, *Mesocricetus auratus*, and *Marmota marmota* have stronger binding ability to the RBD of pangolin-CoV GD, especially *Capra hircus*. This suggests that *Manis javanica* is not the best host of pangolin-CoV GD, even it was separated from *Manis javanica*.

Some earlier published articles claimed that snake may be an intermediate host [18], while it might deviate from epidemiology and evolution. Our results indicated that for both reptiles, like *Ophiophagus Hannah* and *Crocodylus porosus*, and birds, like *Phasianus colchicus* and *Meleagris gallopavo*, their ACE2 showed significant lower binding ability to SARS-CoV-2 spike RBD than mammals, therefore they are unlikely to be the intermediate hosts of SARS-CoV-2.

From the perspective of experimental animals, ferrets, guinea pig, rats, and mice are not good models of SARS-CoV-2, for their ACE2 showed much lower binding ability to spike RBD compared to that of human. Primates, *Mesocricetus auratus* and *Capra hircus* are more suitable to be used as experimental animals for SARS-CoV-2 infection models. These results are also consistent with a recent study in which golden Syrian hamster could be easily infected by SARS-CoV-2 [21]. Our results also showed that dogs are not susceptible to SARS-CoV-2 and also unsuitable for experimental animal model.

Virus traceability is very important for the interpretation of the interpersonal transmission law and evolution history of viruses, and the understanding of the complete chain of viruses from natural hosts to intermediate hosts, and then to the humans. Our work predicted the potential natural and intermediate hosts for SARS-CoV-2, which might contribute to the epidemic prevention and control of COVID-19.

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Supplementary Table 1 Binding free energy of bat coronavirus RaTG13 RBD with

ACE2 from different species calculated by protein-protein docking

NO.	Species Name	Similarity	Accession Number	Free binding energy ($KJ.mol^{-1}$)
1	*Homo sapiens*	100%	NP_001358344.1	-44.9803
2	*Gorilla gorilla*	99.01%	XP_018874749.1	-42.7332
3	*Macaca nemestrina*	95.34%	XP_011733505.1	-42.6326
4	*Papio anubis*	95.34%	XP_021788732.1	-42.6162
5	*Macaca fascicularis*	95.21%	XP_005593094.1	-42.6172
6	*Macaca mulatta*	95.21%	ACI04556.1	-42.8581
7	*Aotus nancymaae*	92.17%	XP_012290105.1	-42.5036
8	*Equus przewalskii*	86.90%	XP_008542995.1	-40.1971
9	*Ceratotherium simum*	85.77%	XP_004435206.1	-41.1406
10	*Panthera tigris* ssp. *altaica*	85.70%	XP_007090142.1	-40.7855
11	*Puma concolor*	85.59%	XP_025790417.1	-40.7563
12	*Panthera pardus*	85.47%	XP_019273508.1	-41.7507
13	*Ictidomys tridecemlineatus*	85.38%	XP_005316051.3	-42.2544
14	*Felis catus*	85.22%	NP_001034545.1	-41.5772
15	*Lynx pardinus*	85.22%	VFV30336.1	-39.4012
16	*Oryctolagus cuniculus*	85.14%	XP_002719891.1	-42.3481
17	*Marmota marmota*	84.88%	XP_015343540.1	-43.0272
18	*Urocitellus parryii*	84.76%	XP_026252505.1	-41.1093
19	*Marmota flaviventris*	84.76%	XP_027802308.1	-41.4861
20	*Manis javanica*	84.76%	XP_017505746.1	-43.2112
21	*Chinchilla lanigera*	84.72%	XP_013362428.1	-37.1876
22	*Fukomys damarensis*	84.72%	XP_010643477.1	-41.4333
23	*Jaculus jaculus*	84.63%	XP_004671523.1	-44.3856
24	*Heterocephalus glaber*	84.60%	XP_004866157.1	-43.38
25	*Octodon degus*	84.47%	XP_023575315.1	-37.3313
26	*Mesocricetus auratus*	84.26%	XP_005074266.1	-44.7522
27	*Arlito syrichta*	84.10%	XP_008062810.1	-37.389
28	*Canis lupus dingo*	84.01%	XP_025292925.1	-35.2498
29	*Nyctereutes procyonoides*	84.01%	ABW16956.1	-37.77
30	*Ursus maritimus*	83.92%	XP_008694637.1	-33.7685
31	*Ursus arctos*	83.88%	XP_026333865.1	-35.5917
32	*Vulpes vulpes*	83.63%	XP_025842512.1	-34.3498
33	*Microtus ochrogaster*	83.63%	XP_005358818.1	-41.3675
34	*Canis lupus familiaris*	83.50%	NP_001158732.1	-38.8909
35	*Paguma larvata*	83.48%	Q56NL1.1	-37.1641
36	*Equus asinus*	83.40%	XP_014713133.1	-39.2759
37	*Ailuropoda melanoleuca*	83.38%	XP_002930657.1	-36.5644
38	*Crocuta crocuta*	83.35%	KAF0878287.1	-37.8297
39	*Vicugna pacos*	83.35%	XP_006212709.1	-35.3267

40	*Camelus ferus*	83.23%	XP_006194263.1	-38.2449
41	*Phodopus campbelli*	82.87%	ACT66274.1	-43.316
42	*Mustela putorius*	82.74%	NP_001297119.1	-35.8347
43	*Balaenoptera acutorostrata*	82.48%	XP_028020351.1	-38.3849
44	*Rattus norvegicus*	82.37%	NP_001012006.1	-39.0555
45	*Grammomys surdaster*	82.24%	XP_028617961.1	-42.3484
46	*Sus scrofa domesticus*	81.94%	ACT66265.1	-40.7439
47	*Mus musculus*	81.86%	NP_001123985.1	-38.9799
48	*Capra hircus*	81.74%	NP_001277036.1	-47.6838
49	*Ovis aries*	81.74%	XP_011961657.1	-43.7355
50	*Pteropus alecto*	81.49%	XP_006911709.1	-42.4567
51	*Mastomys coucha*	81.38%	XP_031226742.1	-39.664
52	*Sus scrofa*	81.37%	NP_001116542.1	-41.5093
53	*Rhinolophus pearsonii*	81.37%	ABU54053.1	-34.2089
54	*Bos mutus*	81.37%	XP_005903173.1	-41.6701
55	*Camelus dromedarius*	80.87%	KAB1253106.1	-39.7657
56	*Rhinolophus macrotis*	80.87%	ADN93471.1	-43.8471
57	*Tupaia chinensis*	80.75%	XP_006164754.1	-36.6856
58	*Miniopterus natalensis*	80.75%	XP_016058453.1	-36.8746
59	*Rhinolophus sinicus*	80.62%	ADN93475.1	-39.9513
60	*Rhinolophus landeri*	80.62%	ALJ94034.1	-38.814
61	*Pteropus vampyrus*	80.62%	XP_011361275.1	-39.0766
62	*Loxodonta africana*	80.50%	XP_023410960.1	-38.0833
63	*Rhinolophus alcyone*	80.50%	ALJ94035.1	-39.366
64	*Rhinolophus ferrumequinum*	80.50%	ADN93470.1	-39.491
65	*Eptesicus fuscus*	80.42%	XP_008153150.1	-36.0798
66	*Myotis brandtii*	80.37%	XP_014399782.1	-41.6428
67	*Rhinolophus pusillus*	80.35%	ADN93477.1	-37.6987
68	*Myotis lucifugus*	80.25%	XP_023609437.1	-36.6078
69	*Cavia porcellus*	79.54%	ACT66270.1	-33.4454
70	*Orycteropus afer*	79.38%	XP_007951028.1	-38.5732
71	*Myotis davidii*	79.15%	XP_006775273.1	-39.3552
72	*Rousettus leschenaultii*	79.13%	ADJ19219.1	-37.6318
73	*Dasypus novemcinctus*	79.13%	XP_004449124.1	-42.4187
74	*Erinaceus europaeus*	79.01%	XP_007538670.1	-41.1565
75	*Rousettus aegyptiacus*	78.88%	XP_015974412.1	-38.1481
76	*Pipistrellus abramus*	76.45%	ACT66266.1	-36.9656
77	*Phascolarctos cinereus*	71.48%	XP_020863153.1	-36.9936
78	*Crocodylus porosus*	67.45%	XP_019384827.1	-41.7424
79	*Phasianus colchicus*	66.09%	XP_031451919.1	-31.6362
80	*Struthio camelus*	65.01%	XP_009667495.1	-38.8162
81	*Ophiophagus hannah*	56.91%	ETE61880.1	-29.8054
82	*Meleagris gallopavo*	55.50%	XP_019467554.1	-38.8765

Supplementary Table 2 Binding free energy of Pangolin-CoV GD RBD with ACE2 from different species calculated by protein-protein docking

NO.	Species Name	Similarity	Accession Number	Free binding energy ($KJ.mol^{-1}$)
1	*Homo sapiens*	100%	NP_001358344.1	-48.0341
2	*Gorilla gorilla*	99.01%	XP_018874749.1	-44.7128
3	*Macaca nemestrina*	95.34%	XP_011733505.1	-44.0687
4	*Aotus nancymaae*	92.17%	XP_012290105.1	-41.4306
5	*Puma concolor*	85.59%	XP_025790417.1	-41.496
6	*Panthera pardus*	85.47%	XP_019273508.1	-42.4629
7	*Ictidomys tridecemlineatus*	85.38%	XP_005316051.3	-44.4516
8	*Oryctolagus cuniculus*	85.14%	XP_002719891.1	-44.3818
9	*Marmota marmota*	84.88%	XP_015343540.1	-45.7725
10	*Urocitellus parryii*	84.76%	XP_026252505.1	-42.6924
11	*Manis javanica*	84.76%	XP_017505746.1	-43.5113
12	*Chinchilla lanigera*	84.72%	XP_013362428.1	-40.2995
13	*Fukomys damarensis*	84.72%	XP_010643477.1	-42.681
14	*Mesocricetus auratus*	84.26%	XP_005074266.1	-47.3596
15	*Paguma larvata*	83.48%	Q56NL1.1	-37.1826
16	*Crocuta crocuta*	83.35%	KAF0878287.1	-27.2922
17	*Capra hircus*	81.74%	NP_001277036.1	-49.4663
18	*Rhinolophus sinicus*	80.62%	ADN93475.1	-42.6029

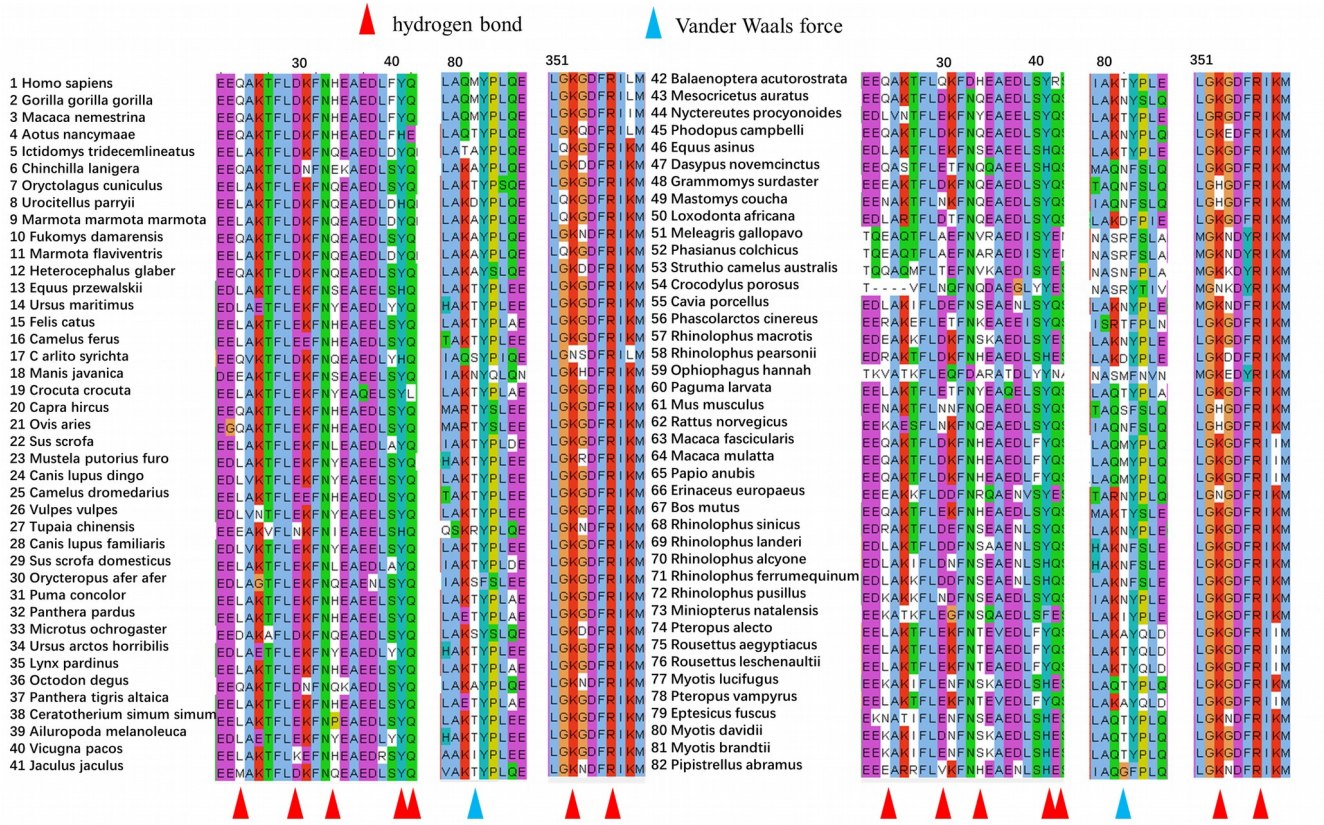

Figure S1. Sequence alignment of ACE2 from 82 species, in which amino acids bound to SARS-CoV-2 Spike-RBD through hydrogen bonds were marked with red triangles below, and amino acids bound to Spike-RBD through van der Waals force were marked with blue triangle below.