Is H34 of ACE2 an indicator of the susceptibility to SARS-CoV-2? Clues from multi-species sequence analyses

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Abstract

SARS-CoV-2, the causal agent of the globally spreading COVID-19, is capable of infecting variable animals besides human being. We evaluated the potential susceptibility of important livestock, pets and aquatic mammals by performing a multi-species sequence analysis of ACE2 based on the reported affected and unaffected animals. We identified a triple amino acid pattern of ACE2, at position 30, 31 and 34, that might be associated with SARS-CoV-2 infection and H34 might be an indicator of the susceptibility to COVID-19.

Keywords:

SARS-CoV-2, COVID-19, Angiotensin-converting enzyme 2, susceptibility, livestock, aquatic mammals

The on-going pandemic COVID-19 continues to wipe throughout the world with rapidly increasing affected cases. It is caused by novel severe acute respiratory syndrome–coronavirus 2 (SARS-CoV-2), a sister virus to SARS-CoV. Angiotensin-converting enzyme 2 (ACE2) is widely expressed in many species and has been found to be the crucial cellular receptor utilized by the SARS-CoV[1] and the SARS-CoV-2[2] for binding and entering cells. The receptor binding domain (RBD) in the S1 region of the virus spike (S) protein in SARS-CoV-2 can bind to the peptidase domain (PD) of ACE2, leading to the fusion of the viral and cellular membranes to gain cell entry[3]. The entry of SARS-CoV-2 into human cell lines can be blocked by an antibody against human ACE2, demonstrating that ACE2 on the host cells is indispensable for the virus invasion.

Current reports showed that human being is not the only species suffered from SARS-CoV-2 attack. Up to date, several kinds of animals have been reported for their reaction to SARS-CoV-2 (Table 1). In Jan 2020, the authors identifying ACE2 as the key receptor for SARS-CoV-2[2] predicted that mice and rat ACE2 might not serve as receptors of SARS-CoV-2. Meanwhile, Zhou et al.[4] expressed ACE2 of different origins on ACE2-free human Hela cells, and found that SARS-CoV-2 was able to entry the cells expressing human, bat, cat and pig ACE2, but not the cells with mouse ACE2[4], indicating that cat and pig might be vulnerable, while mouse may not. Later, the immunity of mice to SARS-CoV-2 was confirmed by two studies. Boudewijns[5] found limited and transient SARS-CoV-2, while Bao[6] found no detectable virus load, in the lungs of inoculated mice. The natural infection of cats and dogs, as the most

popular pets, is not rare. These include a Belgium cat with respiratory symptoms, nausea, and diarrhea[7], two dogs[8] and one cat[9] in Hong Kong with no symptoms, two cats in New York with respiratory symptoms[10], and a study showing around 15% of tested cats in Wuhan were seropositive for SARS-CoV-2[11]. Soon, another experimental study in Tokyo[12] showed that cohoused cats can be infected by inoculated cats, which developed antibodies and no symptoms. A inoculation study[13] found that the detection of SARS-CoV-2 was high in ferrets and cats, low in dogs, but not found in pigs, chickens, and ducks. Especially, the virus can transmit among cats through respiratory droplets[13]. As a close specie to ferret, minks have been confirmed with natural infections with various signs, including respiratory symptoms, at two farms in the Netherlands[14]. Syrian hamsters are also found[5,15] highly reactive and consistently infected with multiple organs affected, with weight loss, tachypnea, and can transmit the virus to naive companions[15]. Inoculated rhesus monkeys, as non-human primates, showed similar symptoms like humans, including weight loss, elevated rectal temperature, reduced appetite, interstitial pneumonia, tachypnea, and dyspnea[16,17]. In addition, four tigers and three lions in New York Bronx Zoo caught SARS-CoV-2 and showed signs of cough and lost appetite, while another tiger got asymptomatic infection[18].

All of the mentioned animals express ACE2. The affinity of S protein for ACE2 is critical for virus transmissibility and disease severity[19]. Since ACE2 is highly conservative in mammals[20], it is likely that more mammals are at risk of SARS-CoV-2 attack than we are already aware of. In particular, we are concerned about mammals

closely related to human beings, such as livestock, pets, and marine mammals. Therefore, we aim to analyze the structure of ACE2 of the concerned animals, using current knowledge of animal susceptibility as a background information, to explore for the key indicator of the level of vulnerability to SARS-Cov-2. Based on this, we further postulate which other animals may be susceptible to SARS-CoV-2. To achieve this, we downloaded the ACE2 protein sequences of 24 mammals from NCBI (Table S1). Besides human, this group of animals consists of typical livestock animals (e.g. sheep and cattle), aquatic mammals (e.g. whale, seal and sea lion), and pets (e.g. hamster, cat, dog, rabbit), etc.

We compared the full length ACE2 protein sequences of these 23 animals with human ACE2 (hACE2). These sequences share high similarity and the global identical rates range from 81.2% to 85.7% except for a higher similarity in rhesus monkey (94.9%) and a lower similarity in cattle (78.6%) (Figure 1A). Out of these results, the identical rates for the reported susceptible animals constitute four of the five species with the highest identity to humans.

We then looked at the α1-helix of ACE2, which serves as an anchor binding to the groove formed by an extended loop of the S-RBD and plays a critical role during the virus-receptor interaction[19,21,22]. We extracted the amino acid sequences of the α1-helix (equivalent to the fragment of aa19 – aa54 of hACE2) and aligned these sequence segments. The result (Figure 1B) shows that the identical rates to human ACE2 range from 74.3% to 91.4% except for the rhesus monkey (100%). As expected, the α1-helix sequences are very similar among those evolutionally closed

animals (Table S2). For examples, cattle, wild yaks and goats have identical α1-helix and very similar to that of sheep (97.1% identity). Interestingly, among the non-primate animals, the α1-helix of cattle, yak, goat and sheep are the most similar to that of human (with 91.4% identity) and higher than those reported susceptible non-primate animals (e.g. cats and tigers, etc.).

The virus-receptor interaction and the affinity of the virus-receptor complex are largely relied on key amino acid residues of the S-RBD and ACE2 that contact with each other. Lan and colleague proposed 20 amino acid residues of ACE2 that contact with S-RBD[21]. We, therefore, extracted these 20 residues from ACE2 of the 24 animals and performed analysis (Figure 2A). As shown in the figure, most of the 20 residues were evolutionally conservative. Relatively high variations were found at residue 24, 30, 34 and 82, containing 4 or 6 different amino acids, and mainly located in the α1-helix of ACE2. Interestingly, these four residues belongs to two of the three clusters of the contact bridge in the hACE2-RBD interface: D30 and H34 locate in the middle of the bridge and interact with K417 and Y453 of S-RBD respectively, while Q24 and M82 locate at one end of the bridge and interact with Q474 and F486 of S-RBD through H-bond or van der Waals forces respectivley[19]. The variations of amino acid residues at these positions might be related to the susceptibility of a specie to SARS-CoV-2. The position 34 is the most non-conservative among these species, and six different amino acids were observed at this position. As for the other three variable positions of ACE2, although four different amino acid residues were identified respectively at these positions, they are actually more conservative than position 34.

Apart from mouse, which has a specific pattern than the others, most of the other species have a leucine or glutamine at position 24, a glutamic acid at position 30 and a threonine at position 82.

We classified the reported animals into three categories. High risk group (HRG) includes human, monkeys, cats, dogs, hamsters, tigers and ferrets. Nil risk group (NRG) includes mouse. As for pig, since in a cell study[4] pig ACE2 has been shown to enable cell entry of the virus, but in an animal study[13] the virus cannot propagate in inoculated pigs, pig is classified into low risk group (LRG). Then, we compared the residues at the 20 positions between HRG and NRG trying to spot the positions displaying biggest differences. We found that for the majority of the 20 positions, HRG and NRG shared some similar choices of amino acids. For example, at position 24, glutamine appears in both NRG and HRG, which renders it no potential for susceptibility prediction. Importantly, a distinct pattern was found at position 30, 31 and 34, where the HRG shares a pattern of 'a negative charged residue at position 30 (i.e. D or E) – lysine at position 31 – a polar residue at position 34 (e.g. H, Y and Q)', while none in the NRG fits in this pattern. As suggested by 3D structural analyses, residues D30, K31 and H34 of hACE2 located in the middle of the α1-helix and formed strong polar contacts with residues K417, E484 and Y453 of S-RBD[22] and such solid interaction networks could reinforce the interaction and binding of S-RBD and ACE2[19].

It is worth notice that, as shown in Figure 2A, the majority of the HRG has a histidine at position 34 (i.e. H34). The three exceptions are ferrets, dogs and hamsters.

Ferrets and dogs have a tyrosine at position 34 (Y34) and reported susceptible to the virus[13]. However, in ferrets SARS-CoV-2 could only replicate in the upper respiratory tract but not in other organs, while infected dogs are asymptomatic[23] and the replication of virus is low[13]. Their results suggested that the susceptibility of ferrets to SARS-CoV-2 was lower than that of cats[13]. According to this observation and given the relative conservativeness of position 30 and 31 of ACE2, we hypothesized that H34 of ACE2 might serve as an indicator for the susceptibility to SARS-CoV-2, although the role of H34 plays during virus infection or entry to host cell is still unclear.

Histidine is unique among the 20 standard amino acids, of which the imidazole side chain has a pK_a of approximately 6. It can easily switch between a proton donor and a proton receptor under physiological conditions and consequently is relevant to the active sites of many enzymes[24]. In the complex of S-RBD and ACE2, H34 locates in the middle of α1-helix and interacts with Y453 of the RBD to reinforce the interaction of the complex[19,22]. The replacement of H34 might interfere the stability of the interaction between S-RBD and ACE2. As mention above, six different amino acids (i.e. H, Y, R, S, Q and L) were found at position 34 among the 24 species. Among them, both histidine and tyrosine have an aromatic polar side chain, which is able to form different types of H-bond (e.g. OH··O/OH··N and OH··φ) that presents an important class of stabilizing interactions. Either type of H-bonds formed by histidine are stronger than that by tyrosine[25]. The side chain of arginine contains a highly polar, positively charged guanidinium group and is capable of forming H-bond

and salt bridge with another polar residue. Serine and glutamine have polar uncharged side chains and are capable of from OH··O/OH··N H-bonds, but the strengths of these H-bonds are weaker than those of histidine and tyrosine. Leucine is a nonpolar amino acid and has relatively week interaction with other residues. This is consistent with the reported low risk of pig to SARS-CoV-2 infection, which has a leucine residue at position 34.

Based on the above analyses, we assumed that the polarity of the residue at position 34 of ACE2 or the strength of the H-bond between the residue 34 of ACE2 and Y453 of S-RBD might associate with the susceptibility of SARS-CoV-2. As for the other animals have not been reported, the important livestock animals (such as cattle, yaks, goats and sheep) have a histidine at position 34 of ACE2 as cats and tigers; and in addition, both the 20 contact residues and the sequence of α1-helix are highly similar to those of hACE2 (Table 1 and 2), which might imply a high susceptibility of these animals. For those aquatic mammals, the ACE2 of whales and dolphins have a histidine or arginine at position 34, and they have more similarity to hACE2 than dogs or ferrets in both the 20 contact residues and α1-helix, which might also indicate certain degree of susceptibility of whales and dolphins. Although the similarity of seals ACE2 to hACE2 is lower than dogs or ferrets, they have a tyrosine at position 34, so the potential risk of infection by SARS-CoV-2 might also exists.

The survival of SARS-CoV-2 is robust in environment, proved by the fact that live SARS-Cov-2 could still be separated from the environmental samples collected from the South China Seafood Market at 11 days after the closure and disinfection.

Later, SARS-Cov-2 was found to survive for a long time in vitro especially on metal, glass or plastic surface[26]. Noteworthy, SARS-Cov-2 was found in one garbage truck besides on 22 market stalls. This raises the concern of the need to strengthen kitchen garbage collection and processing, since urban stray animals depend on leftover food in garbage to survive. Tocsin should be rung about the potential risk of transmission of SARS-CoV-2 to stray animals. Pet is another group of animal worth attention. It is possible for the virus to spread from infected humans to companion animals as reported[8-10], or affected animals to their owners. Furthermore, the infected animals may spread the virus to other animals when they have social activities. The alarm of the spread of SARS-CoV-2 within animal groups is not empty, as a relatively large scale infection of animals have already been reported in Zhang's study[11], where among 102 serum samples collected from cats with confirmed or suspected exposure history in Wuhan, 14.7% tested positive for binding the RBD of SARS-CoV-2, and 10.8% developed neutralizing antibodies. The first animal-to-human transmission of the virus has also appeared recently[27], where three workers on the farms reported with mink infection[14] have been tested positive[27]. Livestock is also a potential group of concern, because most livestock are kept in high density, so large-scale transmission of the virus among livestock is easy to occur. In our analysis, sheep, goat and cattle might be vulnerable to SARS-CoV-2, although further investigations are required.

SARS-CoV-2 can also survive in water. The infectivity of coronavirus TGEV and MHV in clean water at 25°C can last for 22 and 17 days, respectively[28]. In US, higher-than-expected levels of SARS-CoV-2 was detected in sewage in

Massachusetts[29]. In Paris, 4 out of 27 samples of non-drinking tap water were tested positive for SARS-CoV-2[30]. These evidences suggest the risk of transmission through water. This also reminds us that without proper disinfection procedure, aquatic animals, including marine animals, may be exposed to SARS-CoV-2. In our analysis, whales and dolphins might be susceptible, while seals to a lesser extent, to SARS-CoV-2. Considering the fact that the most developed cities are coastal cities, it would be a real ecological catastrophe if marine mammals are proved to be susceptible. The only console might be that there is no evidence on whether novel coronavirus can survive with infectivity in seawater.

Methods

In this study, 24 mammals (Table S1), including important livestock and aquatic mammals, were selected from the ACE2 Orthologs database in NCBI[31] and their ACE2 protein sequences were retrieved from the GenBank[32] using the E-utilities[33,34]. The similarity among these sequence were conducted by multiple sequence alignment using Clustal Omega[35] on EMBL-EBI web server[36]. The fragments of α1-helix (the counterpart of residues 19 to 54 of human ACE2) and the 20 residues contacted with the spike protein receptor binding domain were extracted based on the above multi-sequence alignment results.

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Figure legends:

Fig 1. Percent identity of full length ACE2 (A) and α1-helix of ACE2 (B) to hACE2.

Fig 2. A: The 20 contact residues of ACE2 at the ACE2/S-RBD interface, and B: the ACE2 residues at position 30, 31 and 34 of the reported affected or unaffected animals. CB dolphin: common bottlenose dolphin; LP whale: long-finned pilot whale; PW dolphin: Pacific white-sided dolphin

Table 1. Animals reported for their reactions to SARS-CoV-2.

Species	Type	Time	Location	Findings
Bat	Research[4]	/	/	Bat ACE2 enables cell entry of the virus.
Cat	Research[4]	/	/	Cat ACE2 enables cell entry of the virus.
	News[7]	Mar 27	Belgium	One cat tested positive, and showed respiratory symptoms, nausea, and diarrhea.
	News[9]	Mar 31	Hong Kong	One cat tested positive for virus nucleic acid, but was asymptomatic.
	News[10]	Apr 22	New York, US	Two cats tested positive and had respiratory symptoms.
	Research[11]	/	/	Around 15% of tested cats in Wuhan shows seropositive.
	Research[13]	/	/	Cats showed effective replication of the virus in multiple organs and can transmit to naïve cats.
	Research[12]	/	/	Inoculated cats could pass the virus to cohoused cats, which developed antibodies and no symptoms
Tiger and Lion	News[18]	Apr 25	New York, US	Four tigers and three lions tested positive and showed signs of cough and lost appetite, while another tiger had asymptomatic infection.
Ferret	Research[13]	/	/	Ferrets showed effective replication of the virus in only upper respiratory track, fever and loss of appetite.
Mink	News[14]	Apr 23	Netherlands	Mink being raised on 2 fur farms showed gastrointestinal symptoms and dyspnea.
Monkey	Research[16]	/	/	Experimental infected Rhesus macaques showed weight loss, elevated rectal temperature, reduced appetite, increased respiration rate, interstitial pneumonia.
	Research[17]	/	/	Experimental infected Rhesus macaques showed tachypnea, and dyspnea
Pig	Research[4]	/	/	Pig ACE2 enables cell entry of the virus.
	Research[13]	/	/	Pigs showed no detected replication of the virus.
Hamster	Research[5]	/	/	Hamsters were highly permissive to the virus and developed bronchopneumonia.
	Research[15]	/	/	Hamsters showed consistently infection with multiple organs affected, with weight loss, tachypnea, spleen and lymphoid atrophy, and can infect naïve companions.

Species	Type	Time	Location	Findings
Dog	Research[8]	/	Hong Kong	2 dogs tested positive
	Research[13]	/	/	Dogs showed moderate replication of the virus.
Chicken	Research[13]	/	/	Chickens showed no detected replication of the virus.
Duck	Research[13]	/	/	Ducks showed no detected replication of the virus.
Mouse	Research[4]	/	/	Mouse ACE2 cannot enable cell entry of the virus.
	Research[5]	/	/	Mouse had restricted and transient infection of the virus.

A

	Identity (%)
human	100.0
rhesus monkey	94.9
amur tiger	85.7
rabbit	85.2
domestic cat	84.8
Chinese hamster	84.4
dog	83.5
northern fur seal	83.2
Steller sea lion	83.2
California sea lion	83.1
Pacific walrus	83.0
harbor seal	82.7
domestic ferret	82.6
house mouse	82.1
sheep	81.8
goat	81.8
beluga whale	81.5
CB dolphin	81.5
wild yak	81.4
pig	81.4
PW dolphin	81.3
killer whale	81.2
LP whale	81.2
cattle	78.6

B

	Identity (%)
human	100.0
rhesus monkey	100.0
cattle	91.4
wild yak	91.4
goat	91.4
sheep	88.6
beluga whale	88.6
Chinese hamster	88.6
domestic cat	85.7
amur tiger	85.7
rabbit	82.9
CB dolphin	82.9
house mouse	80.0
pig	80.0
killer whale	80.0
LP whale	80.0
PW dolphin	80.0
domestic ferret	77.1
harbor seal	77.1
Steller sea lion	77.1
California sea lion	77.1
Pacific walrus	74.3
northern fur seal	74.3
dog	73.5

Fig 1.

A

Position	24	27	28	30	31	34	35	37	38	41	42	45	82	83	330	353	354	355	357	393	Identity (%)
human	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	100
rhesus monkey	Q	T	F	D	K	H	E	E	D	Y	Q	L	M	Y	N	K	G	D	R	R	100
cattle	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	90
wild yak	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	90
goat	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	90
sheep	Q	T	F	E	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	90
beluga whale	Q	T	F	Q	K	H	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	90
Chinese hamster	Q	T	F	D	K	Q	E	E	D	Y	Q	L	N	Y	N	K	G	D	R	R	90
domestic cat	L	T	F	E	K	H	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	80
amur tiger	L	T	F	E	K	H	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	80
rabbit	L	T	F	E	K	Q	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	80
CB dolphin*	R	T	F	Q	K	R	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	80
killer whale	R	T	F	Q	K	R	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	80
LP whale*	R	T	F	Q	K	R	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	80
PW dolphin*	R	T	F	Q	K	R	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	80
pig	L	T	F	E	K	L	E	E	D	Y	Q	L	T	Y	N	K	G	D	R	R	80
dog	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	G	D	R	R	75
domestic ferret	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	R	D	R	R	70
harbor seal	L	T	F	E	K	Y	E	E	E	Y	Q	L	T	Y	N	K	R	D	R	R	70
California sea lion	L	T	F	E	K	S	E	E	E	Y	Q	L	T	Y	N	K	H	D	R	R	70
Steller sea lion	L	T	F	E	K	S	E	E	E	Y	Q	L	T	Y	N	K	H	D	R	R	70
northern fur seal	L	T	F	E	K	S	E	E	E	Y	Q	F	T	Y	N	K	H	D	R	R	65
Pacific walrus	L	T	F	E	K	Y	E	E	E	Y	Q	F	T	Y	N	K	H	D	R	R	65
house mouse	N	T	F	N	N	Q	E	E	D	Y	Q	L	S	F	N	H	G	D	R	R	65

B

Position	30	31	34	risk
human	D	K	H	HGR
rhesus monkey	D	K	H	HGR
Chinese hamster	D	K	Q	HGR
domestic cat	E	K	H	HGR
amur tiger	E	K	H	HGR
domestic ferret	E	K	Y	HGR
dog	E	K	Y	HGR
pig	E	K	L	LGR
house mouse	N	N	Q	NGR

Fig 2