





Evolutionary Analysis of Mammalian ACE2 and the Key Residues Involved in Binding to the Spike Protein Revealed Potential SARS-CoV-2 Hosts

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ABSTRACT

Introduction: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spilled over to humans via wild mammals, entering the host cell using angiotensin-converting enzyme 2 (ACE2) as receptor through Spike (S) protein binding. While SARS-CoV-2 became fully adapted to humans and globally spread, some mammal species were infected back. The present study evaluated the potential risk of mammals becoming hosts for SARS-CoV-2 through bioinformatics prediction based on ACE2 receptors. **Methods:** We used evolutionary bioinformatic approaches and comparative analysis of ACE2 critical residues that bind SARS-CoV-2 S-protein and predicted potential SARS-CoV-2 hosts among mammals and assessed their risk. **Results:** ACE2 phylogenetic tree placed primates close to rodents and rabbits. Felines, rodents, and rabbits had higher ACE2 similarities than human ACE2 (hACE2). Farmed animals, such as bovines, swine, and equids, had similar ACE2 compared to hACE2; however, these animals showed low SARS-CoV-2 susceptibility. Some cetaceans also presented high similarities in ACE2 key residues with hACE2. **Conclusion:** Here, we showed wild and domestic mammals with a low divergence of ACE2 compared to humans, discussing their possible chance of being infected, especially those animals kept as livestock or pets. Regarding the feasible transmission through contaminated water, cetaceans can be at risk of SARS-CoV-2 infection. Extensive surveillance of SARS-CoV-2 should be applied to prevent new coronavirus outbreaks and preserve mammals from infectious threats.

INTRODUCTION

Coronaviruses have caused severe human infectious diseases since the beginning of the century. Severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) were transmitted through wild animal-to-human spillover, triggering outbreaks [1]. While SARS-CoV and MERS-CoV were limited to some countries, SARS-CoV-2 spread worldwide, infecting hundreds of millions of people, causing the coronavirus disease 2019 (COVID-19) pandemic.

The close contact between humans and animals favored the coronavirus SARS-CoV-2 spillback transmission [2]. The spike (S) protein of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2). The

interaction between the S-protein receptor-binding domain and critical residues of ACE2 enables the virus to enter the host cell. The ACE2 receptor seems to be a specific barrier that interferes with coronavirus spillover. For instance, SARS-CoV-2 should hardly infect particular species due to low efficient binding with ACE2, as observed in murine species [4]. Conversely, the susceptible hosts, such as primates and felines, which present similar ACE2 receptors to human ACE2 (hACE2) receptors, have higher ACE2 binding efficiency with the SARS-CoV-2 S-protein compared to resistant species [5].

Considering that ACE2 similarity can be a relevant factor determining host range and interspecies transmission of SARS-CoV-2 infection, we evaluated the potential risk of various mammals becoming SARS-CoV-2 hosts by applying phylogeny and comparing ACE2

critical residues which bind S-protein. An additional evolutionary approach based on ACE2 complete sequences was performed to extend the analysis on the SARS-CoV-2 hosts. This study provides valuable insights to comprehend the role of new players in SARS-CoV-2 transmission chains.

MATERIAL AND METHODS

Phylogenetic analysis was performed on 124 complete ACE2 protein sequences from mammals, selected from the Protein Database of National Center for Biotechnology Information - NCBI (www.ncbi.nlm.nih.gov/protein/). Protein sequences accession codes are available in Supplementary Table 1 (request via email to jommid@gmail.com). Complete ACE2 amino acid sequences were aligned using MUSCLE [6] through SeaView [7]. The Jones-Taylor-Thornton model [8] with a gamma distribution for among-site rate variation (JTT+G model) was used to

construct a maximum likelihood phylogenetic tree. We used the FigTree v1.3.1 software (http://tree.bio.ed.ac.uk/software/figtree/) to format the phylogenetic tree. The model selection and construction of the phylogenetic tree were conducted using MEGA X software [9]. ACE2 evolutionary divergent pairwise analysis was also estimated based on the number of amino acid substitutions per site, using the amino acid complete sequence-based alignment, through the MEGA X software [9]. Mammals with incomplete ACE2 amino acid sequences were excluded from the analysis. We also performed pairwise analyses based on the number of similar key residues of ACE2 that bind to the SARS-CoV-2 S-protein. Critical residues that imply the binding of ACE2 to the SARS-CoV-2 S-protein were based on previous studies [3, 10]. The key residues of mammalian ACE2 were compared within different taxonomic groups to show conserved amino acids using Skyglign software [11].

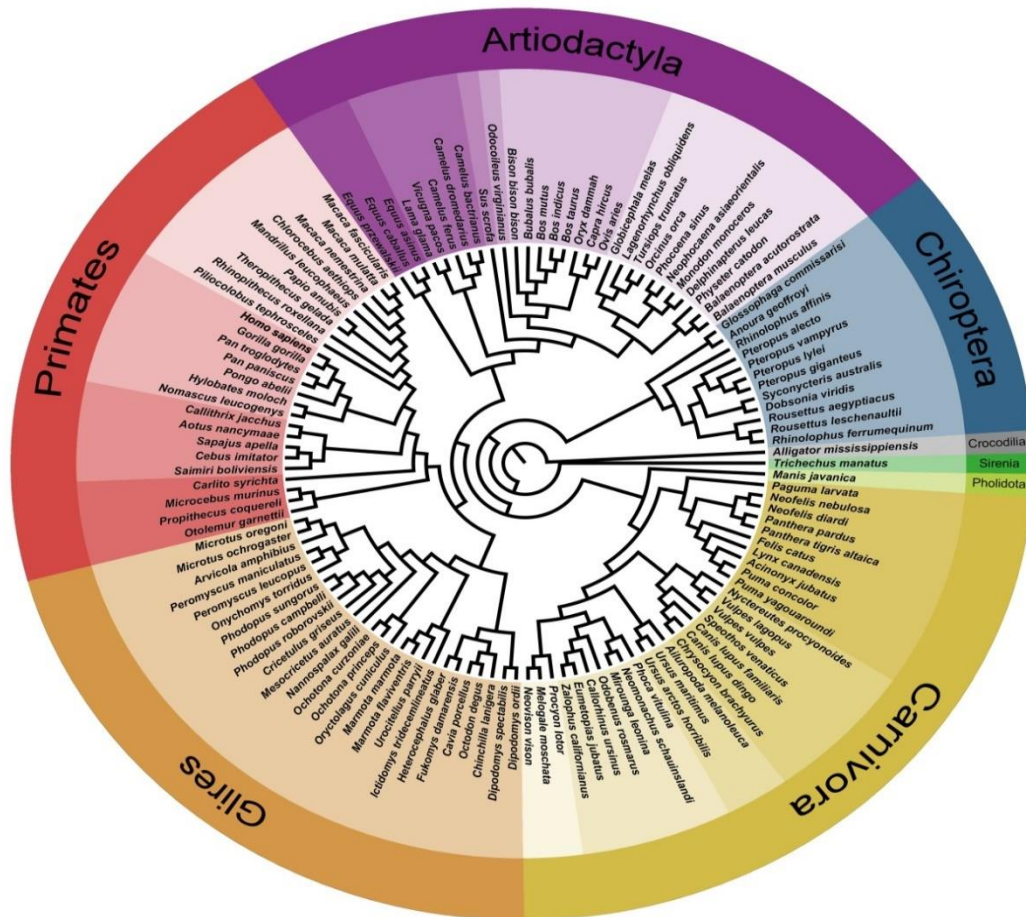


Fig. 1. Mammals ACE2 phylogenetic tree. Maximum likelihood phylogenetic analysis involved 124 amino acid sequences from mammals obtained from the NCBI Protein database (www.ncbi.nlm.nih.gov/protein). The *Alligator mississippiensis* ACE2 was used as an outgroup. Accession codes are in the supplementary material (request via email to jommid@gmail.com). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site.

Analyses of susceptibility prediction based on ACE2

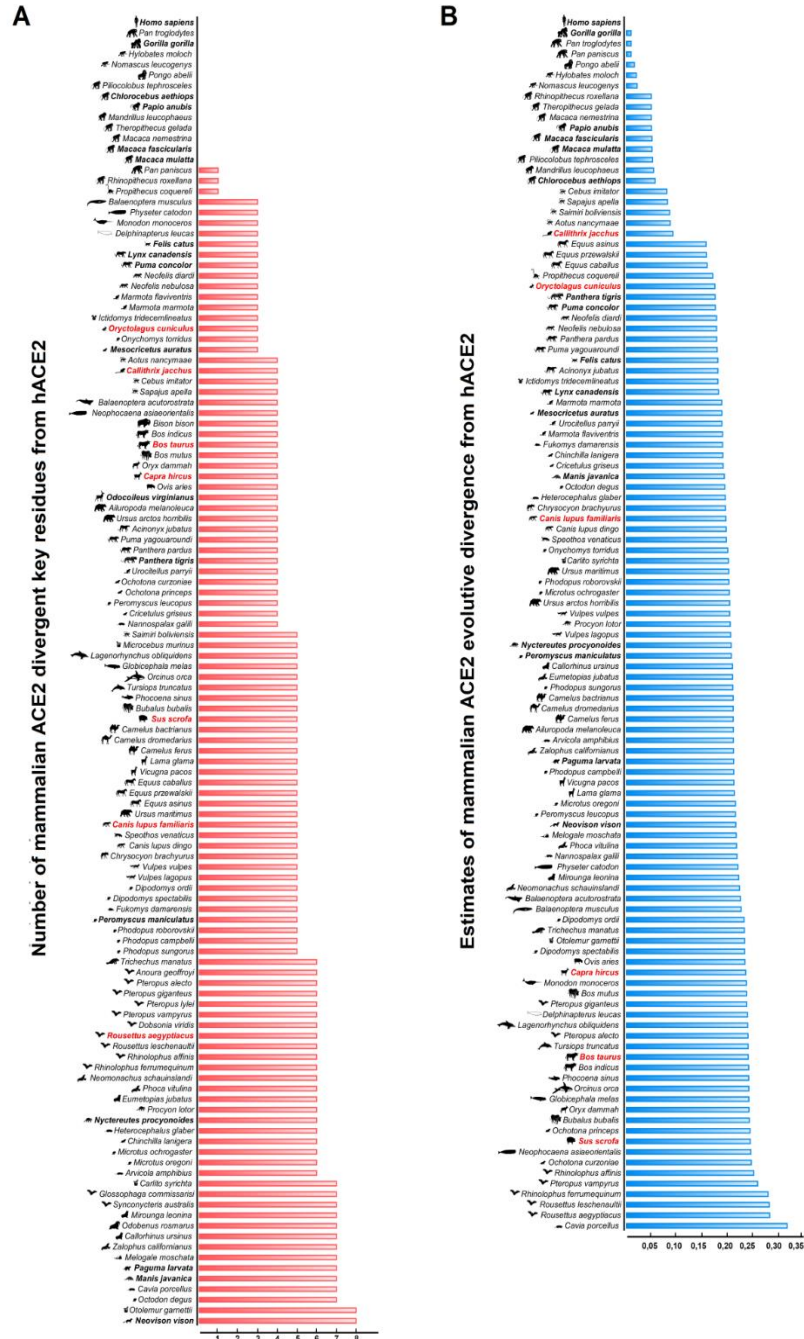


Fig. 2. (A) Number of ACE2 divergent key residues from hACE2 were evaluated through pairwise comparison of 23 residues that interact with the SARS-CoV-2 S-protein. Critical residues that imply the binding of hACE2 to the SARS-CoV-2 S-protein were based on previous studies (references number 3 and 10). This analysis involved 124 amino acid sequences. The alignment of the residues is detailed in the supplementary Figure 1 (request via email to jommid@gmail.com). (B) Evolutionary divergence analysis of mammalian ACE2 protein complete sequences compared to hACE2 consisted of the number of amino acid substitutions per site. This analysis involved 115 amino acid sequences, excluding nine mammals with incomplete ACE2 amino acid sequences. Bold species show SARS-CoV-2-susceptible mammals, and red species are mammals with low susceptibility to SARS-CoV-2. Graphs were generated using Microsoft Excel. Animal icons were obtained from the Noun Project website (thenounproject.com).

RESULTS

According to the ACE2 phylogenetic tree (Fig. 1), humans were placed closest to other non-human primates, such as simians and apes, as expected. ACE2-based phylogeny placed humans closer to gorillas than chimpanzees (*Pan troglodytes*), which means that this tree did not represent a species tree. Glires (rodents and rabbits), a large group including some SARS-CoV-2-susceptible hosts, was the closest animal group to primates. Artiodactyla, carnivores, and Chiroptera formed monophyletic groups, respectively. Malayan pangolin (*Manis javanica*), the probable intermediate host of SARS-CoV-2, appeared next to carnivores. The Chiroptera group emerged next to the root of the tree.

Based on identical key residues from hACE2 and primates' ACE2, apes and Old World monkeys showed the highest identity (Fig. 2A), whereas New World monkeys and lemurids presented some different residues. The New World monkeys presented two ACE2 taxon-specific mutations, Q42E and G354Q (Fig. 3). Lemurids presented higher variability within the primates. However, for Coquerel's sifakas (*Propithecus coquereli*), the ACE2 key residues were highly identical to those of hACE2 (Fig. 2A). Similarly, the ACE2 evolutionary analysis (Fig. 2B) showed great apes, monkeys, and Coquerel's sifakas closest to humans. However, the other lemurids had high evolutive divergence rates.

In the carnivore group, felids presented the most similar ACE2 key residues to hACE2, although canids and ursids had a few differences. The carnivore group includes some known hosts of SARS-CoV-2, such as minks, wild felines, civets, raccoon dogs, and domestic cats and dogs. Although ACE2 of civets, minks, pangolins, and raccoon dogs presented some of the highest numbers of different key residues from hACE2 (Fig. 2A), ACE2 evolutionary analysis (Fig. 2B) estimated that these carnivores had low divergence rates.

Regarding the Artiodactyla group, some cetaceans, such as the blue whale (*Balaenoptera musculus*), beluga (*Delphinapterus leucas*), narwhal (*Monodon monoceros*), and cachalot (*Physeter catodon*), presented three different ACE2 key residues compared to hACE2 (Fig. 2A). White-tailed deer (*Odocoileus virginianus*), a SARS-CoV-2-susceptible host, had only four differences. Bovids had identical ACE2 key residues as white-tailed deer. Camelids and equids had five key ACE2 residues different from those in hACE2. ACE2 evolutionary analysis (Fig. 2B) showed that equids had the lowest divergence within the Artiodactyla group. However, bovids, cetaceans, and pigs had high evolutive divergence rates from humans.

Some Glires species had ACE2 key residues with high identity to hACE2 (Fig. 2A). Rabbits (*Oryctolagus cuniculus*), golden hamsters (*Mesocricetus auratus*), marmots (*Marmota marmota* and *Marmota flaviventris*), squirrels (*Ictidomys tridecemlineatus*), and grasshopper

mice (*Onychomys torridus*) had only three different residues compared to humans. ACE2 evolutionary analysis (Fig. 2B) showed similar results to ACE2 key residues analysis. Both analyses highlighted rabbits, golden hamsters, marmots, squirrels, and grasshopper mice. In contrast, *Chichilla lanigera* and *Cricetulus griseus* (Chinese hamsters), both popular pets, were highlighted by evolutionary analysis, which showed low divergence rates from hACE2.

Bats (Chiroptera) had at least six ACE2 key residues different from hACE2 (Fig. 2A) and presented taxon-specific ACE2 key residues at amino acid positions 34 and 330 (Fig. 3). ACE2 evolutionary analysis included bats as the most divergent group compared to humans (Fig. 2B).

DISCUSSION

Predicting SARS-CoV-2 new hosts can conduct epidemiological surveillances to specific animals and their habitats. Although the prediction based on ACE2 key residues which bind the S-protein can be a helpful approach, we observed that this analysis showed some contradictory points. For instance, some known SARS-CoV-2 hosts presented significant differences from humans. The presented study showed that known SARS-CoV-2 hosts, such as pangolins, minks, civets, and raccoon dogs, had more differences from humans than some animals with low susceptibility to SARS-CoV-2 infection, such as bovids. Thus, we considered implementing a concomitant approach based on the evolutionary divergence of ACE2 to corroborate the analysis of the key residues. The ACE2 evolutive divergence analysis included pangolins, minks, civets, and raccoon dogs closer to humans than the ACE2 key residues analysis did.

Furthermore, some species with low SARS-CoV-2-susceptibility, presenting few differences in ACE2 key residues from hACE2, showed high evolutive divergence rates with humans. This means that ACE2 based-evolutionary analysis could enhance the prediction of SARS-CoV-2-susceptible mammals. Therefore, combining both ACE2 analyses can also improve the interpretation of the potential SARS-CoV-2-susceptible hosts.

Regarding the close evolutionary relationship, primates represent the animal group with the highest risk for SARS-CoV-2. In this context, Old World monkeys, such as the green monkey (*Chlorocebus aethiops*), Rhesus monkey (*Macaca mulatta*), cynomolgus monkey (*Macaca fascicularis*), and baboon (*Papio anubis*), are susceptible to SARS-CoV-2 infection, shed viral RNA, and develop lung injuries and pneumonia [12–14]. We showed that monkeys and humans shared identical key residues in ACE2 (Fig. 2A) and had the lowest evolutive divergence from humans. Conversely, marmoset (*Callithrix jacchus*), a New World monkey, appeared to be relatively less susceptible to SARS-CoV-2, presenting mild infections [13]. The evolutionary distance between

marmosets and humans and their dissimilar ACE2 key residues (Figs. 1 and 2) suggested that the S-protein may bind marmoset ACE2 less efficiently than hACE2. Moreover, New World monkeys have specific mutations at some positions in ACE2 regions that can alter the

ACE2 analysis predicting the risk of hosts to SARS-CoV-2 interaction with S-protein (Fig. 3). Consequently, no sign of SARS-CoV-2 infection in free-living marmoset was documented in Brazil, which sustains a high SARS-CoV-2 transmission [15].

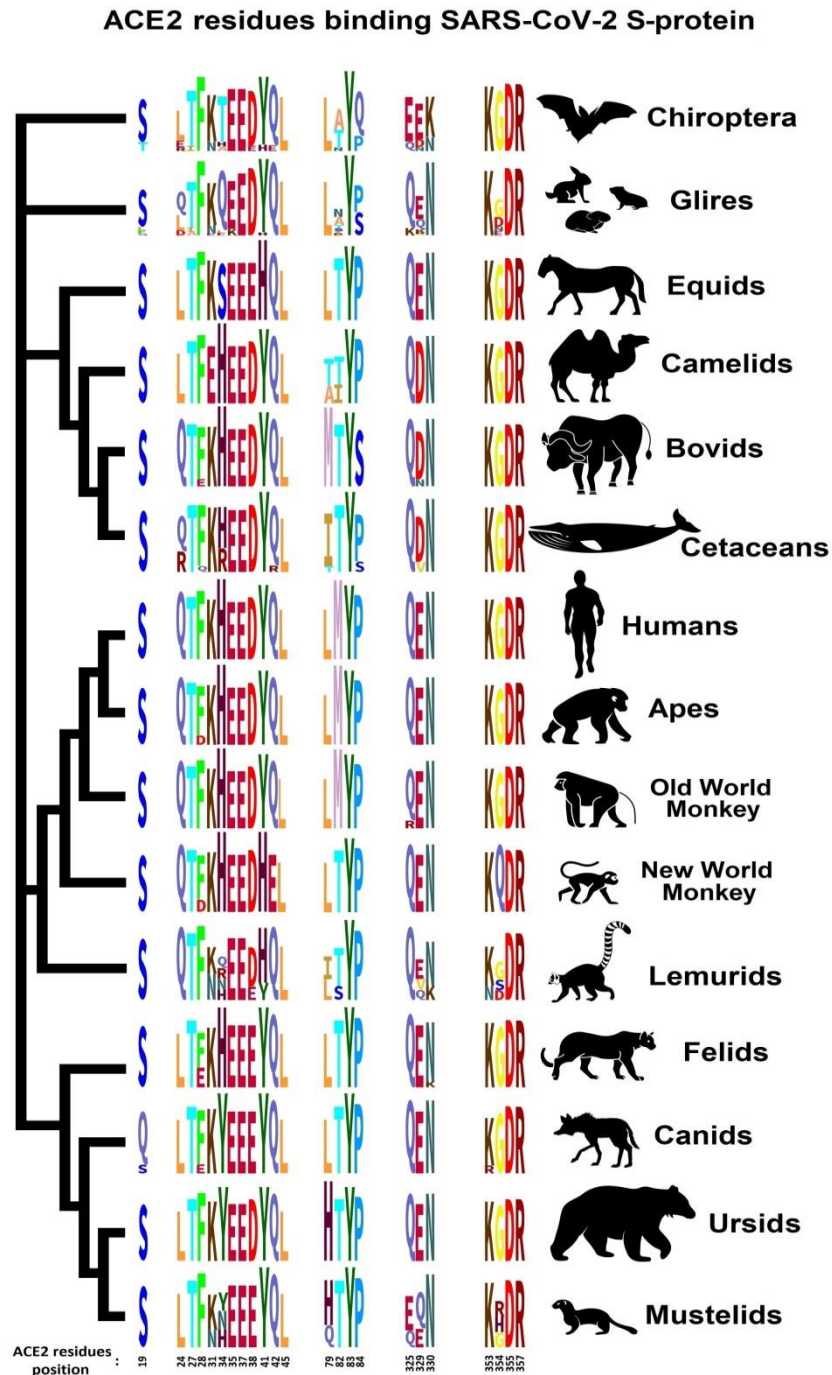


Fig. 3. Sequence logo visualization of ACE2 key residues which interact with the SARS-CoV-2 S-protein from different mammalian taxonomic groups were performed using Skylign software (11). Animals icons were obtained from the Noun Project website (thenounproject.com).

Regarding the lemurids, despite the phylogenetic distance from humans, the Coquerel's sifakas (a lemur of the sifaka genus *Propithecus*) ACE2 key residues presented high identity to those of hACE2 (Fig. 2A), and the evolutive divergence between this species and humans was low (Fig. 2B). The deforestation caused by humans has increased exposure and interactions of wild lemur populations with humans and domestic animals [16,17]. Therefore, ACE2 identity and the human disturbance put Coquerel's sifakas at the risk of SARS-CoV-2 infection.

The Felidae encompass a group that also demands concern. It is noteworthy that civet, a species closely related to felines, was implicated as a possible host in the emergence of SARS-CoV [18]. The analysis of the ACE2 key residues showed few differences between Felidae and humans and low evolutionary distance rates (Fig. 2). SARS-CoV-2 infects felines efficiently [19] and causes clinical manifestations and histopathologic lesions similar to acute COVID-19 [20]. Tigers and lions are SARS-CoV-2-susceptible hosts and can be infected naturally [21–23]. Captive felines were tested positive for SARS-CoV-2 after contact with SARS-CoV-2-infected zoo workers [21]. For instance, viral RNA was detected in nasal samples from lions for approximately two weeks after the onset of clinical signs of SARS-CoV-2 infection [23].

Cats have elevated expression of ACE2 among various organs [24]. SARS-CoV-2 RNA detected in the upper respiratory tract revealed the potential ease of transmission via respiratory droplets [19]. Infected cats shed high levels of SARS-CoV-2 RNA [25]. Domestic and wild felines release viral RNA through feces [19, 22, 23, 26], favoring the alternative the fecal-oral transmission route.

Additionally, felines were involved in a transmission chain with the American minks (*Neovison vison*), mustelid. Minks were likely to be infected by farmworkers. Within a few days, a significant number of farmed minks can become infected with SARS-CoV-2 [27], spreading SARS-CoV-2 to stray cats in the vicinity of infected mink farms [28] and back to humans [29]. While ACE2 key residues comparative analysis showed that mink ACE2 was distant from hACE2, the ACE2 evolutionary analysis showed the minks closer to humans. Thus, the lower evolutive divergence could better explain the success of SARS-CoV-2 spillover from humans to minks (Fig. 2). Minks and cats shared more identical ACE2 key residues than humans and minks or humans and cats (Fig. 3 and Supplementary Figure 1). Felines and mustelids expressed elevated levels of ACE2 in the lungs, resulting in high susceptibility to SARS-CoV-2 infection (30).

The susceptibility to SARS-CoV-2 of species with broad populations, such as farmed animals, demands concern. For instance, white-tailed deer, a SARS-CoV-2-susceptible host, can be infected naturally, shedding

infectious virus through nasal secretion and fecal samples [31, 32]. A portion of white-tailed deer in some North American states presented antibodies against SARS-CoV-2, showing that the virus has circulated among deer in their habitat [31]. Deer have a high population density and are kept as livestock [33]. Their ecological interactions place these animals at an important position in the SARS-CoV-2 transmission chain [34]. Interestingly, the present study showed that deer and bovids share identical ACE2 key residues (Fig. 2) and a close phylogenetic relationship (Fig. 1). Conversely, there is no evidence that bovids and swine shed SARS-CoV-2 RNA [35, 36]. This fact can be explained by the high evolutive divergence between the farmed mammals ACE2 with hACE2. However, frequent contact with diverse SARS-CoV-2 variants could turn bovids and swine into efficient hosts. For instance, mice showed no susceptibility to SARS-CoV-2 when experimentally inoculated with an ancestral strain [4], but mice infected with SARS-CoV-2 gamma variant strain presented viral replication in the lungs [37]. Furthermore, the higher prevalence of SARS-CoV-2 infection among farmworkers than non-farm workers [38] could contribute to cross-species transmission involving humans and cattle or other farmed mammals.

Within the Artiodactyla group, Camelids and Equids presented five different ACE2 key residues from hACE2. SARS-CoV-2 appeared to have no capacity to bind Camelids ACE2; however, further studies are necessary to confirm this issue. Our study revealed that equids presented the lowest evolutive divergence rates from hACE2 among the non-primates mammals. Although equids did not appear to be susceptible hosts, constant epidemiological surveillance should be applied, considering the low evolutive divergence between horse ACE2 and hACE2.

Dogs also have low susceptibility to SARS-CoV-2 and appear not to support viral replication well [19]. ACE2 key residues of canids, compared with hACE2, showed low differences (Fig. 2A), which should favor susceptibility of canids to SARS-CoV-2. Naturally-infected domestic dogs of infected owners have been described [39]. Instead, domestic dogs experimentally infected with SARS-CoV-2 did not shed viral RNA or develop the clinical disease [40]. Regarding wild canids, no signs of SARS-CoV-2 infection in free-living red foxes (*Vulpes vulpes*) were found [41]. At the same time, the raccoon dog (*Nyctereutes procyonoids*), a known SARS-CoV host, exhibited seroconversion, efficient transmission, and high SARS-CoV-2 RNA shedding in nasal, oropharyngeal, and rectal samples [42].

Surveillance of carnivore mammals can be an essential means to detect infected wildlife exposed to SARS-CoV-2. Carnivores are the mammalian group with the highest number of known SARS-CoV-2-susceptible hosts. Phylogenetically, Malayan pangolins (*M. javanica*), the probable intermediate hosts during SARS-CoV-2 emergence, appeared next to carnivores.

Predatory SARS-CoV-2-susceptible mammals could provide opportunities for viral spillover into wild carnivore populations. Our results also highlighted ursids in the potential transmission chain of SARS-CoV-2 due to the equal number of ACE2 key residues with hACE and their low evolutive divergence rate (Fig. 2). Large populations of ursids share diverse landscapes with humans, requiring testing the susceptibility of these carnivores to SARS-CoV-2.

Some carnivores such as foxes, raccoon dogs, and minks occur at low density or solitarily, limiting the onward transmission of SARS-CoV-2 in these species. However, these carnivore species are also kept as livestock, providing opportunities for viral transmission.

Rabbits are another farmed animal susceptible to SARS-CoV-2. However, the transmission between rabbits seemed to be less efficient than infection within hamsters [43]. The present study showed that rabbits and hamsters shared a high identity in ACE2 key residues and low evolutive divergence from hACE2 (Fig. 2). Rodents and rabbits encompass a large and heterogeneous group. Some species, kept as pets, remain in permanent contact with their owners, while others are widely distributed in the wild and are often preys to other animals. These scenarios placed these animals at a potential position in the SARS-CoV-2 transmission chain to humans or other wild mammals. Furthermore, according to the ACE2 phylogenetic tree, rabbits and rodents (Glires) are closest to humans and other primates (Fig. 1). This finding might reinforce the possibility of a putative SARS-CoV-2 transmission chain, including these animals.

An additional group demanding particular concern is the Chiroptera. Bats appear to be unique in their capacity to harbor persistent viral infections. SARS, MERS, and COVID-19 involved bats in their transmission chains. Analyses based on the ACE2 showed that bats had at least six different key residues and high evolutive divergence rates from hACE2 (Fig. 2). Bats have taxon-specific mutations (Fig. 3), which must be implicated as potential barriers to spillover to humans, suggesting a need for intermediate hosts during the emergence of SARS-CoV-2, as well as barriers to the spillover back to bats. However, these issues need to be confirmed by additional experiments. Despite the differences in the ACE2 key residues, the possible involvement of bats in the SARS-CoV-2 spillback chain should not be underestimated.

In the present study, the identity of cetaceans ACE2 key residues with hACE2 indicated that aquatic mammals could be at risk. Although ACE2 evolutionary analysis did not show cetaceans close to humans, some cetaceans had only three different ACE2 key residues compared to hACE2. Hence, the susceptibility of cetaceans and marine carnivore mammals to the SARS-CoV-2 and the virus viability under environmental conditions and the fecal-oral transmission must be evaluated. SARS-CoV-2 has not yet been detected in marine mammals, while

Gammacoronavirus occur in cetaceans [48]. Although the enveloped viruses are considered fragile, coronaviruses present potential environmental resistance. SARS-CoV appears to survive for four days in diarrhetic feces [44], and SARS-CoV-2 RNA has been detected in sewage and wastewater [45]. Previously, a study detected infectious particles of animal coronaviruses, which persisted in water and sewage for days [46]. In this context, pathogens of fecal origin were already detected up to 8 km from an ocean sewage outfall [47]. Altogether, these facts emphasize SARS-CoV-2 surveillance in aquatic mammals.

The COVID-19 pandemic is a global health crisis that continues to spread, especially in countries with low vaccination rates, spanning diverse habitats. The SARS-CoV-2 susceptibility of mammal species has elevated the complexity of the COVID-19 pandemic. The large population of some susceptible animals kept as livestock, pets, or in the wild and the increased number of SARS-CoV-2 infections in humans comprises a complex network. The potential to infect mammals represents a rapid capacity of coronavirus to adapt to new susceptible hosts. Consequently, the broad surveillance of SARS-CoV-2 in mammals with low divergence in the ACE2 receptor compared to hACE2 should be implemented to prevent a new coronavirus emergence.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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