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# **Molecular and functional characterization of Sika deer ACE2 as a receptor for SARS-CoV-2**

**A short running title: Sika deer ACE2 as SARS-CoV-2 receptor**

**Subject section: Virology**

**Specified field: Animal RNA virus**

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#### **Data availability statement**

For other information, please contact the corresponding author.

#### **Disclosure**

The authors declare no conflicts of interest.

## Abstract

Surveillance of SARS-CoV-2 in white-tailed deer (WTD, *Odocoileus virginianus*) has revealed its widespread and sustained transmission across North America, with evidence suggesting possible transmission from deer to humans. In the following surveillance studies in other deer species, however, little evidence of infection spread was found, including in sika deer (*Cervus nippon*) in our previous study. Differences in the structure of the virus entry receptor angiotensin-converting enzyme 2 (ACE2) are known to act as one of the functional barriers to SARS-CoV-2 infection. To investigate the molecular basis of the lack of SARS-CoV-2 transmission to sika deer, we performed structural and functional analyses of the sika deer ACE2 in comparison with WTD ACE2. Comparison of sika deer ACE2 sequence and those of cervid species with WTD ACE2, followed by *in silico* molecular dynamics analysis, revealed a substitution of lysine to asparagine in position 31 commonly found in cervid ACE2s can potentially alter binding to the SARS-CoV-2 spike (S) protein receptor-binding domain (RBD). Functional assays in cells expressing sika deer and WTD ACE2s showed minimal differences in viral binding and replication, demonstrating that SARS-CoV-2 can similarly utilize ACE2 from both species. These findings suggest that sika deer and possibly other cervids may be highly susceptible to SARS-CoV-2 and highlight the need to investigate other factors impacting virus spread in deer populations.

**Key words:** ACE2; COVID-19; sika deer; SARS-CoV-2; white-tailed deer; wildlife.

## Introduction

SARS-CoV-2, the virus causing COVID-19 in humans, has been shown to affect various species of domestic and wild animals, with numerous cases reported in white-tailed deer (WTD, *Odocoileus virginianus*) in Canada [1,2] and across multiple states in the US [3,4,5,6,7], where nearly 10% of the animals were reported positive for the virus genome [6]. The SARS-CoV-2 strains detected in WTD were shown to be closely related to those circulating in humans [6]. In addition, variants that had long ceased circulating in the human population were detected in deer, suggesting transmission from humans and onward transmission between deer [6]. Indeed, the deer-to-deer transmission was observed both in experimental [8,9] and natural settings [6]. Moreover, suspected human infections with SARS-CoV-2 potentially originating from wild deer were reported [2]. These findings highlight the risk for extinct viral variants to persist in deer populations and re-emerge in humans [10], creating serious public health concerns.

Following the discovery of SARS-CoV-2 widespread infections in WTD, several studies were conducted to assess SARS-CoV-2 transmission in widely distributed deer species in Europe. In Poland, red deer (*Cervus elaphus*) tested negative [11], as did red deer, roe deer (*Capreolus capreolus*), and fallow deer (*Dama dama*) in Austria and Germany [12]. A large-scale survey conducted in the UK between January 2020 and May 2021 tested six deer species (i.e. Chinese Water deer (*Hydropotes inermis*), fallow deer, muntjak (*Muntiacus reevesi*), red deer, roe deer, sika deer (*Cervus nippon*), and sika/red deer hybrid) and found no signs of SARS-CoV-2 transmission [13]. Similarly, in Asia, our findings showed no infections in sika deer in northern Japan [14]. However, between 2020 and 2022, while roe deer still tested negative,

fallow deer and red deer were found seropositive in Spain, with most cases from the deer inhabiting a suburban park near Madrid [15]. Furthermore, recently elk (*Cervus elaphus*) in the US [16] and fallow deer from a large urban park in Ireland were found seropositive [17]. Although these findings suggest that SARS-CoV-2 can infect multiple deer species, reported cases remain few and have primarily involved animals with a high risk of human contact. The stark contrast between studies conducted in Europe/Japan and North America may reflect differences in host species and environmental conditions across the studied deer populations, including human-deer contact rates, deer feeding practices, the prevalence of SARS-CoV-2 during surveillance, circulating virus strains, and the surveillance methods used. Clarifying the role of species-specific susceptibility is essential to understanding the potential for SARS-CoV-2 transmission and persistence in wild deer.

An important determinant of viral susceptibility is the presence and accessibility of cellular receptors that mediate virus binding and entry. A receptor for SARS-CoV, angiotensin-converting enzyme 2 (ACE2), has become widely known for its role as a host cell receptor for SARS-CoV-2, too [18,19,20]. SARS-CoV-2 binds to ACE2 via the receptor-binding domain (RBD) region of the spike (S) protein and undergoes proteolytic activation, followed by virus entry into the cell [21]. In animals, the ACE2 sequence similarity to human ACE2 has been widely used to predict potential animal hosts for the virus [22]. As such, WTD ACE2 was shown to be highly similar to human ACE2 and to bind multiple SARS-CoV-2 RBDs *in vitro*, suggesting the high susceptibility of WTD to SARS-CoV-2 infections [23]. In other deer species, ACE2 sequence alignment and structural comparison showed a high degree of homology between them [20,22,24]. Although these results suggest the susceptibility to SARS-CoV-2 in different deer species, lysine (N) to asparagine (K) at position 31 (namely K31N) in the ACE2 of species in genus *Cervus* has been proposed to contribute to their reduced viral susceptibility [13,24]. This relates to the important role that K31 plays in the interaction between human ACE2 and SARS-CoV-2 [25,26]. Although the K31N substitution has been suggested to hinder viral binding, its effects in *Cervus* species have not been investigated *in vitro*, and few studies have assessed these deer species' susceptibility *in vivo* [27].

In this study, we conducted sequencing analysis, protein structure modeling, and functional assays to compare the ACE2 receptors of sika deer and WTD. We aimed to explore the possible contributions of differences in host viral entry receptor to the absence of SARS-CoV-2 transmission in sika deer in Japan and potentially in other deer species. Our findings demonstrate that there are limited functional differences between sika deer and WTD ACE2 to serve as receptors for SARS-CoV-2, while the K31N substitution was also found in sika deer ACE2. This suggests that other host and environmental factors might influence the susceptibility of deer species to SARS-CoV-2 infections, highlighting the need for continued monitoring of the virus in cervids.

## Materials and Methods

### Sika deer sample collection

Hokkaido sika deer (*Cervus nippon yesoensis*) were either culled as part of population management, euthanized due to injury or illness, or captured for ongoing ecological research between March 2022 and December 2024 as described previously [28], where the individuals from March 2022 to February 2023 were overlapped with our previous survey [14]. Blood was collected from euthanized deer during necropsy, either from the chest cavity, jugular vein, or heart. Serum was separated and stored at  $-80^{\circ}\text{C}$ . Nasopharyngeal swabs were taken from both nostrils using cotton-tipped swabs, placed in tubes containing serum-free DMEM, and stored at  $-80^{\circ}\text{C}$ . RNA from deer nasal swabs and other liquid samples in this study was extracted using NucleoSpin RNA Virus kit (TAKARA Bio Inc., Shiga, Japan).

The sampling was performed according to the "Act on the Protection and Management of Animals" and the "Regulations on Animal Experiments at Hokkaido University" and approved by the Animal Care and Use Committee of Hokkaido University (permit No. 22-0016).

#### **Cells and viruses**

HEK293T cells, Vero-TMPRSS2 cells [29], and Hokkaido sika deer (*Cervus nippon yesoensis*) primary fibroblast cells (National Institute of Environmental Studies (NIES), Japan) (Supplementary Table 1) were grown at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in Dulbecco's Modified Eagle Medium (DMEM) (Nacalai Tesque Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; ICN Biomedicals Inc.), 1% Penicillin/Streptomycin, and 1% L-glutamine. DMEM supplemented with 2% FBS was used to culture cells infected with SARS-CoV-2.

A SARS-CoV-2 strain WK-521 (GISAID ID: EPI\_ISL\_408667; PANGO lineage A) and a SARS-CoV-2 Omicron strain (TY38-871) (GISAID ID: EPI\_ISL\_7571618; PANGO lineage BA.1.1) were obtained from the Japan Institute for Health Security (JIHS) (formerly National Institute of Infectious Diseases (NIID), Japan). The viruses were propagated in Vero-TMPRSS2 cells and stored at  $-80^{\circ}\text{C}$  until use.

#### **SARS-CoV-2 quantitative RT-PCR (RT-qPCR)**

RT-qPCR was performed as described previously [14]. Briefly, the reaction was performed with the One Step PrimeScript III RT-qPCR Mix (TAKARA Bio Inc.), N2 Primer/Probe mix (TAKARA Bio Inc.), and 5  $\mu\text{l}$  of sample RNA in a 20  $\mu\text{l}$  reaction volume. The cycling conditions consisted of one cycle at  $25^{\circ}\text{C}$  for 10 min, one cycle at  $52^{\circ}\text{C}$  for 2 min, and 45 cycles of  $95^{\circ}\text{C}$  for 10 sec, followed by an extension at  $60^{\circ}\text{C}$  for 30 sec. Serial 10-fold dilutions of the Positive Control RNA Mix (TAKARA Bio Inc.), with a minimum detectable level of 100 copies per well, were used to quantify the viral gene copy number.

#### **SARS-CoV-2 seroprevalence in wild sika deer**

Enzyme-Linked Immunosorbent Assay (ELISA), immunofluorescence assay (IFA), and virus neutralization assay (VNA) for sika deer seroprevalence survey were conducted as described previously [14]. ID Screen SARS-CoV-2 Double Antigen Multispecies ELISA (Innovative Diagnostics, Grabels, France) was used as an initial screening and performed according to the manufacturer's instructions.

Samples that showed S/P ratio greater than or equal to 0.60 were considered positive and subsequently tested using the IFA and VNA. IFA has been performed with Vero-TMPRSS2 cells infected with 100 median tissue culture infectious dose (TCID<sub>50</sub>) of WK-521 or Omicron. Infected cells fixed with 4% paraformaldehyde (PFA) were incubated with diluted ELISA-positive animal serum, followed by antibody detection using Protein A/G conjugated with FITC (BioVision, acquired by Abcam, Cambridge, United Kingdom). To perform VNA, ELISA-positive serum samples were serially diluted and mixed with 100 TCID<sub>50</sub> of WK-521 or Omicron, then added to Vero-TMPRSS2 cells. After 5 days post-inoculation, cytopathic effect (CPE) was assessed using an inverted microscope.

### **Sequencing and prediction of deer ACE2 sequences and multiple sequence alignment**

To determine the sequence of the sika deer ACE2 gene, primers were designed based on the ACE2 gene sequences of elk (*Cervus elaphus*, GenBank ID: XM\_043458543.1) and red deer (*Cervus canadensis*, GenBank ID: XM\_043896107.1). RNA from the lymph node of a Hokkaido sika deer captured in our previous study (Kovba et al., 2024) was extracted using the NucleoSpin RNA Kit (TAKARA Bio Inc.) and used for amplification and sequencing of the ACE2 gene. RT-PCR amplification was performed with PrimeScript One Step RT-PCR Kit Ver.2 (TAKARA Bio Inc.) in a 25 µl reaction volume consisting of 12.5 µl of OneStep RT-PCR buffer, 1 µl of PrimeScript 1 step Enzyme Mix, 0.4µM forward and reverse primer (primers used are listed in Supplementary Table 2), 9.5 µl of DW, and 1 µl of sample RNA. The PCR cycling conditions were as follows: denaturation at 50°C for 30 min; one cycle of elongation at 94°C for 2 min; 35 amplification cycles consisting of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec followed by a final cycle of 72°C for 5 min. Samples were then visualized by electrophoresis on 1.5% agarose gels containing MIDORI Green (NIPPON Genetics Co., Ltd., Tokyo, Japan). DNA from the gel was purified using NucleoSpin Gel and PCR Clean-up (TAKARA Bio Inc.), eluted in 16 µl of water, and stored at -20°C. DNA was later sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) in the 3500xL Genetic Analyzer (Thermo Fisher Scientific). The obtained sequence was aligned to red deer ACE2 using Molecular Evolutionary Genetics Analysis version 11 (MEGA11) [30] for constructing the sika deer ACE2 gene sequence.

For determining cervid ACE2 gene sequences not available in the public database, transcriptome reads for each cervid species were downloaded from the National Center for Biotechnology Information (NCBI) database [31], trimmed, and mapped to the sika deer ACE2 gene sequence using QIAGEN CLC Main Workbench 23.0 (QIAGEN, Aarhus, Denmark). The consensus sequences of mapped reads were extracted, and putative ACE2 sequences of cervid deer were constructed. Predicted ACE2 sequences and their original sequencing reads are listed in the Supplementary Table 3, together with other sequences used in the present study. These sequences were translated to amino acid sequences and then aligned to WTD and sika deer ACE2 sequences together with other ACE2 sequences obtained from GenBank using the Clustal W alignment tool [32] on MEGA11.

### **ACE2/RBD interaction visualization**

White-tailed deer ACE2 in complex with the prototype SARS-CoV-2 spike protein receptor-binding domain (RBD) and Omicron RBD were obtained from the RCSB Protein Data Bank (RCSB PDB) (PDB ID: 8HG0 and 8IFZ, respectively). K31N mutation to mimic sika deer ACE2 structure was introduced using the MUTATE function in the Swiss PDB Viewer [33], and the structure was visualized with PyMOL (The PyMOL Molecular Graphics System, Version 3.0, Schrödinger, LLC).

#### **Prediction of the interaction between deer ACE2 and SARS-CoV-2 S protein RBD**

To determine whether any of the substitutions in peptidase domain of ACE2 in sika deer and WTD affect binding between sika deer ACE2 and the SARS-CoV-2 RBD, we performed molecular dynamics (MD) simulations using the Desmond simulation package in Schrödinger Suite 2022-4 (Schrödinger, LLC, New York, USA). Mutant structures containing N31K or K31N substitutions were generated using the Maestro module in Schrödinger Suite 2022-4. Energy minimization and MD simulations were conducted with the OPLS4 force field. Each system was dissolved in 0.15 M NaCl supplemented simple point charge (SPC) water model. Following system minimization and equilibration, 100 ns production simulations were run under NpT ensemble conditions at 310 K and 1 bar, using a Nose–Hoover thermostat. For each complex, three independent simulations were conducted with different initial velocity distributions. Protein-protein interactions were evaluated using the Protein Contacts tool in Molecular operating environment (MOE) software version 2022.02 (Chemical Computing Group, Montreal, QC, Canada). For each system, 500 trajectory frames were sampled at 100 ps intervals from the final 50 ns for contact analysis. Residue pairs that formed interactions in at least 30% of the frames were considered significant.

#### **ACE2 plasmid construction**

cDNA of Hokkaido sika deer mRNA was synthesized using PrimeScript II 1st strand cDNA Synthesis Kit (TAKARA Bio Inc.) according to the manufacturer's instructions from the lymph node RNA used in the above sequencing, and the DNA fragment for cloning into a mammalian expression vector (pCAGGS) was amplified from the cDNA using forward and reverse primers containing 15-base-pair (bp) overlapping regions to the pCAGGS vector (Supplementary Table 2) by using KOD One PCR Master Mix -Blue (TOYOBO Co., Ltd., Osaka, Japan). The complete WTD ACE2 sequence predicted in this study, with the addition of the 15 bp pCAGGS vector overlap regions on each end of the sequence, was synthesized with gBlocks Gene Fragments (Integrated DNA Technologies Inc., Coralville, Iowa, U.S.). PCR-amplified sika deer ACE2 and synthesized WTD ACE2 were respectively cloned into the pCAGGS vector using the In-Fusion HD Cloning Kit (TAKARA Bio Inc.). Plasmid DNAs were transformed into ECOS Competent E. coli DH5  $\alpha$  (Nippon Genetics Co., Ltd.) and extracted from the cultured cells using Qiagen Plasmid Plus Midi Kit (QIAGEN). The insert sequences were confirmed using Sanger sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit in the 3500xL Genetic Analyzer.

#### **ACE2 protein expression in HEK293T cells**

Plasmid DNAs were transfected into HEK293T cells using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, plasmid DNAs with twice the volume of Lipofectamine 2000, relative to DNA, were diluted in Opti-MEM reduced serum medium (Thermo Fisher Scientific) and incubated at room temperature for 20 min. After applying the plasmid DNA mixture to the cells, the transfected cells were incubated at 37°C for 4-6 hours, and the medium was exchanged for culture medium (10% FBS-containing DMEM). Cells were used at 1 day post-transfection (dpt) for further analyses.

To confirm exogenous deer ACE2 expression in HEK293T cells, at 1 dpt, cells were fixed with 4% PFA for 24 h at 4°C. Fixed cells were permeabilized with 0.1% Triton X-100 (SIGMA-ALDRICH, St. Louis, Missouri, United States) at room temperature for 15 min, then washed with 1X PBS and stained with mouse anti-ACE2 monoclonal antibodies (Cat# GTX635897, GeneTex, Inc., California, United States), followed by goat anti-mouse Alexa-Fluor 488 IgG (Cat# A32723 or Cat#A-11029, Invitrogen, Thermo Fisher Scientific) or goat anti-mouse Alexa Fluor 594 (Cat# A32742, Invitrogen, Thermo Fisher Scientific) and 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for nuclei staining. Stained cells were visualized using a Zeiss AXIO fluorescence microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany), and images were processed on ZEN Blue 3.4 software (Carl Zeiss Microscopy GmbH).

The expression of deer ACE2 in HEK293T cells was also confirmed by using flow cytometry; cells were detached at 1 dpt with TrypLE protease (Thermo Fisher Scientific) and twice washed in PBS supplemented with 2% FBS. Cells were then fixed with 4% PFA and stained similarly to the IFA. After anti-ACE2 staining, cells' ACE2 expression was analyzed using a BD FACS Flow Cytometer (BD Biosciences, New Jersey, USA).

## **Western Blotting**

Western blotting was performed to confirm deer ACE2 expression in HEK293T cells and primary sika deer cells. Cells were harvested, washed twice with ice-cold PBS containing 2% FBS, and incubated with lysis buffer at 4°C for 30 min (EzRIPA lysis kit #WSE-7420, ATTO, Tokyo, Japan). Samples were centrifuged at 15,000 rpm for 15 min at 4°C, supernatants collected and stored at -80°C till use.

Protein samples were mixed with 5 µl of sample buffer (EzApply, #AE-1430, ATTO), boiled at 95°C for 5 min. Samples were then loaded onto a gel (e-PAGEL HR, #EHR-T/R1020L, 14 well, ATTO) for electrophoresis. Separated proteins were transferred to the polyvinylidene difluoride (PVDF) membrane using the TransBlot Turbo system (Bio-rad, Hercules, CA, USA). Membranes were incubated in Blocking One (Nacalai Tesque Inc.) and stained overnight at 4°C using mouse anti-ACE2 monoclonal antibodies (Cat# GTX635897, GeneTex, Inc.) or rabbit anti-GAPDH monoclonal antibodies (#2118, Cell Signaling Technology, Danvers, MA, USA). Target proteins were visualized with HRP-conjugated anti-mouse antibodies (Cat# ab6789, Abcam) or HRP-conjugated anti-rabbit antibodies (Cat# ab6721, Abcam) and ECL Substrate (QBlot kit M #WSE-4057, ATTO). Images of the membranes were taken on LuminoGraph III (ATTO).

## **Infection with SARS-CoV-2**

HEK293T cells expressing sika deer or WTD ACE2 or the primary cells were infected with serially diluted WK-521 and Omicron. Cells were incubated at 37°C for 1 h and washed twice with 1xPBS, then cultured with DMEM containing 2% FBS for 7 days. Daily, each well was observed for CPE using an inverted microscope. For confirmation of the absence of infectious virus in the supernatant of the infected primary cells, the supernatant was passaged to the same or Vero-TMPRSS2 cells, followed by RT-qPCR, IFA, or observation of CPE.

For detection of SARS-CoV-2 N in the infected cells, IFA was performed by using the primary rabbit anti-SARS-CoV-2 nucleocapsid (N) polyclonal antibodies (Cat# PA1-41098, Invitrogen, Thermo Fisher Scientific) and secondary anti-rabbit Alexa-Fluor 594 IgG (Cat# A32754, Invitrogen, Thermo Fisher Scientific) as described above.

## **SARS-CoV-2 binding, replication, and production assays**

HEK293T cells with or without transfection of sika or WTD ACE2 plasmid were infected at a multiplicity of infection (MOI) = 10 of WK-521 and Omicron. Cells were incubated at 4°C for 30 min before infection and then incubated with the inoculum for 1 h at 4°C, followed by washing with PBS three times. RNA was extracted from cells using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) and used for RT-qPCR. The amount of viral RNA in cells is considered as the amount of virions attached to the cells (binding). For replication and production assays, infected cells were cultured at 37°C to facilitate virus entry and replication. 24 h after the infection, RNA was extracted from the supernatant and also from the cells. The amounts of viral RNA in the cells and supernatants are respectively considered as the amount of viruses replicating in the cells and produced from the infected cells.

## **Statistical analysis**

RT-qPCR results were log-transformed to meet assumptions on normality and homogeneity of variance and pairwise comparisons were performed using one-way ANOVA with Tukey's multiple comparisons test in GraphPad Prism version 10.4.1 for Windows (GraphPad Software, Boston, Massachusetts, USA, [www.graphpad.com](http://www.graphpad.com)).

## **Results**

### **Surveillance of SARS-CoV-2 infections in sika deer reveals no infection in 2022-2024**

Wildlife surveillance has been continued after our first report in 2022-2023 [14] to determine whether virus transmission had occurred in the following years. Between 2023 and 2024, no infection was observed in any of the tested animals (Table 1). Although more ELISA-positive animals were detected, no animal was positive with the VNA and IFA (data not shown).

## **Molecular comparison among deer ACE2s**

To reveal the role of ACE2 molecules in the susceptibility of *Cervus* deer to SARS-CoV-2, we first compared the ACE2 amino acid sequences of multiple deer species. For deer species whose ACE2 gene sequences are unavailable or only partly available on public datasets, we newly determined them by RT-PCR and Sanger sequencing (for sika deer) and by construction based on publicly available transcriptomic data (for other species) (Supplementary Table 3). ACE2 sequences from various deer species were highly similar to that of white-tailed deer (WTD), with a total of 30 variable residues observed across species. The greatest diversity was found in red deer and Père David's deer (*Elaphurus davidianus*), each differing from WTD in 13 positions, while mule deer (*Odocoileus hemionus*), a close relative of WTD, showed only 2 amino acid differences (Figure 1B). Among the 30 residues where WTD ACE2 is different from at least one of the other deer ACE2s, 15 residues (marked in green) are identical between WTD ACE2 and the human sequence. Interestingly, 3 out of the 15 amino acid positions are well conserved in deer of the genus *Cervus* (i.e., N31, L640, and Y709). There were 7 sites in which the ACE2 of multiple deer resembled human ACE2 but differed in WTD ACE2 (marked in yellow), with all of these residues being located in the peptidase-domain region. Between sika deer and WTD ACE2s, there are 12 amino acid differences.

We then analyzed the role of the 7 residues in the peptidase-domain region in which WTD and sika deer differed, in the interaction with the SARS-CoV-2 WK-521 and Omicron RBD. MD simulation, computer-based analysis of protein interaction based on the physical principles of atomic motion, showed that asparagine substitution at position 31 (K31N) in sika deer can be highly associated with ACE2/RBD binding. We then analyzed the effect of K31N on the binding affinity of WTD ACE2. K31N substitution introduced in WTD ACE2 affected binding to both the wild type (Figure 2A, C, E) and Omicron (Figure 2B, D, F) RBDs. It reduced van der Waals distance at positions F490, L492, and Q493, while enhancing at Y489 (Figure 2C, D), slightly affecting the ACE2-RBD interaction. Hydrogen bonds were reduced at positions L492 and Q493.

## **Functional comparison of sika deer and white-tailed deer ACE2s**

To study the effect of the amino acid sequence difference between sika deer and WTD ACE2s in the ACE2-RBD interaction during virus cell entry, we compared the function of those ACE2 molecules *in vitro*. First, HEK293T cells transiently expressing ACE2 (Figure 3A-C) were infected with WK-521 and Omicron at MOI = 10 (Figure 3D). Co-localization of exogenous deer ACE2 and SARS-CoV-2 N was observed in the infected cells, and most of the SARS-CoV-2 N expression was observed in the cells expressing deer ACE2 (Figure 3D).

To compare the difference between sika deer and WTD ACE2-expressing cells in their ability to facilitate virus entry and replication, we measured virus binding, replication, and production in infected cells (Figure 4A). Binding of SARS-CoV-2 was examined using cells incubated with viruses for 1 hour at 4°C, and it was significantly higher in ACE2-expressing cells compared to HEK293T negative control cells (Figure 4B). To evaluate viral replication and release, we measured viral titers in the supernatant 24 hours

after infection (Figure 4C). Across four independent experiments, viral RNA levels were higher in WTD ACE2-expressing cells. We also quantified intracellular viral RNA to assess replication efficiency (Figure 4D). Results showed slightly higher viral replication and release in WTD ACE2-expressing cells. To control for potential differences in ACE2 expression levels, we calculated the ratio of intracellular viral RNA at 1 dpi ("replication") to bound viral RNA at 0 dpi ("binding") (Figure 4E). This ratio was higher in WTD ACE2-expressing cells, but no statistical significance was observed.

#### **Virus replication evaluation in sika deer primary cells**

In addition to using HEK293T cells transiently expressing exogenous deer ACE2s, we aimed to test whether cells directly obtained from sika deer are susceptible to infection. Primary cells obtained from Hokkaido sika deer were used for this purpose (Supplementary Table 1 and Supplementary Figure 1A). Although cells were exposed to both the WK-521 and Omicron SARS-CoV-2 strains at an MOI of 50-100, no CPE was observed in these cell cultures (results not shown), and viral N protein could not be detected by the immunofluorescence assay after 7 days of culture (Supplementary Figure 1B). In the second passage, the cells also showed no signs of infection. While a small amount of viral RNA was detected by RT-qPCR in the supernatants from the second passage (Supplementary Figure 1C), no Vero-TMPRSS2 cells inoculated with supernatants from both passage 1 and 2 of the primary cultures showed signs of infection (data not shown). Importantly, no evident ACE2 expression was observed in the studied sika deer primary cells that were derived from the cartilage tissue (Supplementary Figure 1D).

#### **Discussion**

A high reproduction number of SARS-CoV-2 in wild white-tailed deer (WTD) in North America [34] contrasts with the limited spread of the virus observed in European deer species [11,12,13]. To assess the potential of sika deer, a widely distributed species in Asia, to act as reservoirs for SARS-CoV-2, we continuously surveyed deer in northern Japan in our previous and present study [14]. Until December 2024, there is no evidence of SARS-CoV-2 transmission in this deer population. To better understand the difference between WTD and sika deer in the field, we employed molecular and functional analyses to investigate the differences in the host viral entry receptor ACE2 among sika deer and WTD, which potentially alter the host susceptibility to SARS-CoV-2 infections.

Our sequence analysis with some newly determined ACE2 amino acid sequences of deer species was in line with previous reports showing high similarity among deer, especially in the sites responsible for SARS-CoV-2 binding [24]. In sika deer ACE2, 7 differences from WTD ACE2 were present in the peptidase domain region, which plays a role in virus binding, and we showed that the sole Asn31 (K31N) substitution might affect the interaction with the virus S protein. K31N may lower the interaction with residues F490, L492, and Q493 of SARS-CoV-2 S, including a reduction in hydrogen bonding at L492 and Q493 in both the prototype and Omicron RBD, indicating a strong influence on the binding at these sites. F490 and Q493 are among the key amino acid residues in the interaction between SARS-CoV-2 S and

human [25] and WTD [23] ACE2s, respectively. However, in our *in vitro* studies, SARS-CoV-2 N expression was largely restricted to cells expressing deer ACE2, indicating ACE2-dependent infection, and no significant differences were observed between sika deer and WTD ACE2-expressing cells in their ability to support viral entry and replication. Taken together, the molecular differences between WTD and the other deer ACE2s may not alter the susceptibility of deer to SARS-CoV-2 infections.

In the present study, we showed that molecular differences in ACE2 do not appear to fully account for the absence of SARS-CoV-2 transmission in sika deer. However, this study only examined the molecular differences of the ACE2 receptor, while its differences in expression levels in the respiratory system may affect the susceptibility to SARS-CoV-2. Indeed, previous studies showed that ACE2 expression could be detected in the respiratory tract of WTD [9,35], fallow deer, roe deer, red deer, and muntjak [36], but none was detected in Chinese water deer (*Hydropotes inermis*), Pere David's deer (*Elaphurus davidianus*), Barasingha (*Rucervus duvaucelii*), or reindeer (*Rangifer tarandus tarandus*) [36]. In the moose (*Alces alces alces*), no ACE2 was observed in the lung, but could be detected in the nasal turbinate and trachea [36]. We did not observe productive virus replication in primary cells derived from sika deer cartilage, as indicated by the absence of virus growth in Vero-TMPRSS2 cells exposed to the primary cell supernatant. The connective tissue origin of these cells, along with the number of passages prior to virus exposure [37], may have contributed to the observed lack of ACE2 expression and virus susceptibility. In addition, other host cellular factors and immune defense factors may limit the virus infection at steps other than virus binding. For example, studies reported the difference between genes expression related to innate immune response in WTD and human respiratory tracheal epithelial cells *in vitro* [38] and in nasal epithelium *in vivo* [39] which may contribute to stronger antiviral response and asymptomatic viral spread in WTD. Moreover, recent virus challenge studies have shown that mule deer (*Odocoileus hemionus*), which is closely related to WTD, is highly susceptible to SARS-CoV-2 infections [8,9,27], while elk, a species in the genus *Cervus*, is also susceptible, but relatively less so compared with mule deer [27], suggesting lower susceptibility of members of the genus *Cervus* to SARS-CoV-2. Further studies using sika deer lung cells, which have been shown to be susceptible to the virus infection in WTD [8] and animal experiments may be required to reveal the mechanism underlying the absence of infections in the sika deer population.

In summary, the minimal binding differences to SARS-CoV-2 between sika deer and WTD ACE2 suggest that ACE2 structural variation, including K31N substitution, is unlikely to significantly suppress SARS-CoV-2 infections in sika deer. Other factors influencing biological susceptibility, as well as ecological and epidemiological conditions, may play a more significant role in shaping SARS-CoV-2 transmission dynamics in wild deer populations. To fully assess the possibility of deer playing a role in SARS-CoV-2 persistence in nature, future research should address discrepancies between existing surveillance studies and expand monitoring efforts to include additional deer species, diverse habitats, and varying degrees of human proximity.

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**Tables**

Table 1. SARS-CoV-2 surveillance in sika deer, Hokkaido, Japan in 2022-2024.

| Test method | Number of animals positive/tested |       |      | Total positive/tested (%) |
|-------------|-----------------------------------|-------|------|---------------------------|
|             | 2022†                             | 2023‡ | 2024 |                           |
| ELISA       | 4/55                              | 2/64  | 0/47 | 6/166 (3.61)              |
| VNA*        | 0/4                               | 0/2   | ND   | 0/6 (0)                   |
| RT-qPCR     | 0/51                              | 0/74  | 0/50 | 0/175 (0.0)               |

† from our previous study (Kovba et al., 2024)

‡ partly from our previous study (Kovba et al., 2024) (45 samples in ELISA and 56 samples in RT-qPCR)

\* only performed with samples tested positive for ELISA

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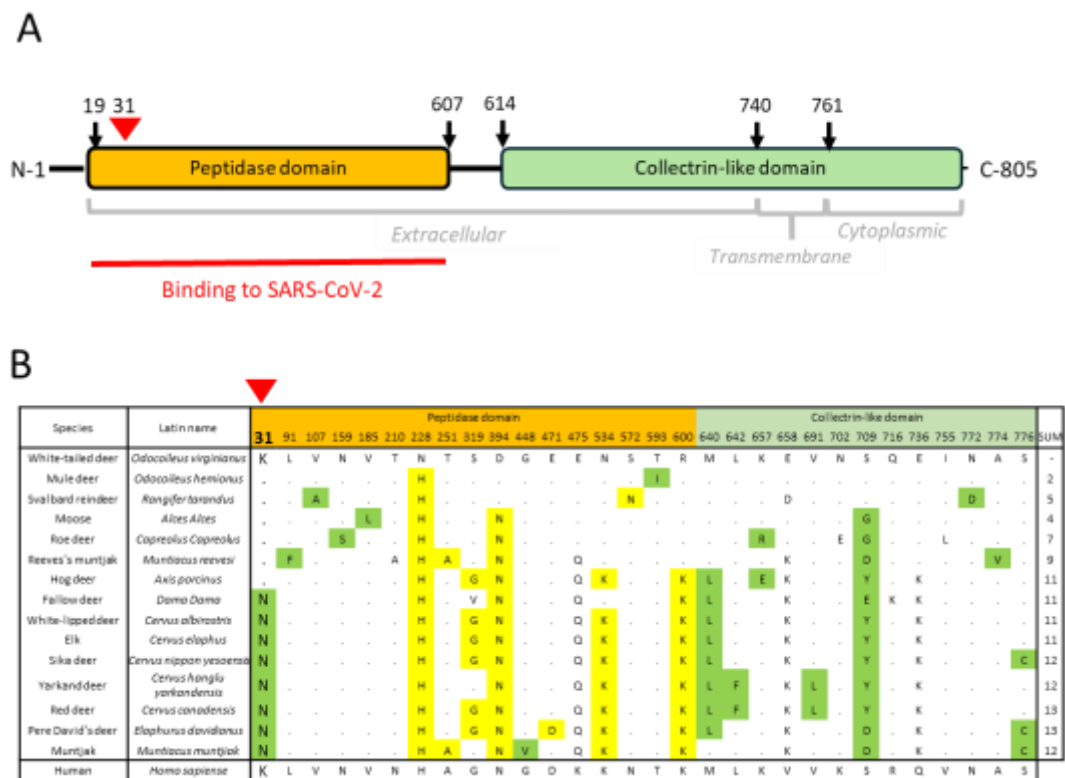


Figure 1. A) Schematic diagram of the ACE2 molecule. ACE2 is a type I membrane protein and consists of two functional domains: a peptidase and a collectrin-like domain. Amino acid positions based on human ACE2 are indicated, and the peptidase domain region responsible for the interaction with SARS-CoV-2 spike (S) protein is shown in red. Site 31 is highlighted with a red arrowhead. B) ACE2 amino acid sequence comparison. The positions where at least one deer ACE2 has different amino acid residues compared with white-tailed deer (WTD) ACE2 are shown. Dots indicate identical amino acids to WTD ACE2. SUM indicates the number of mismatches in the sequence compared to WTD ACE2. Amino acids of deer ACE2s that are different from the identical amino acids between WTD and human ACEs are highlighted in green. Sites where the ACE2s of multiple deer but not WTD resembled human ACE2 are highlighted in yellow.

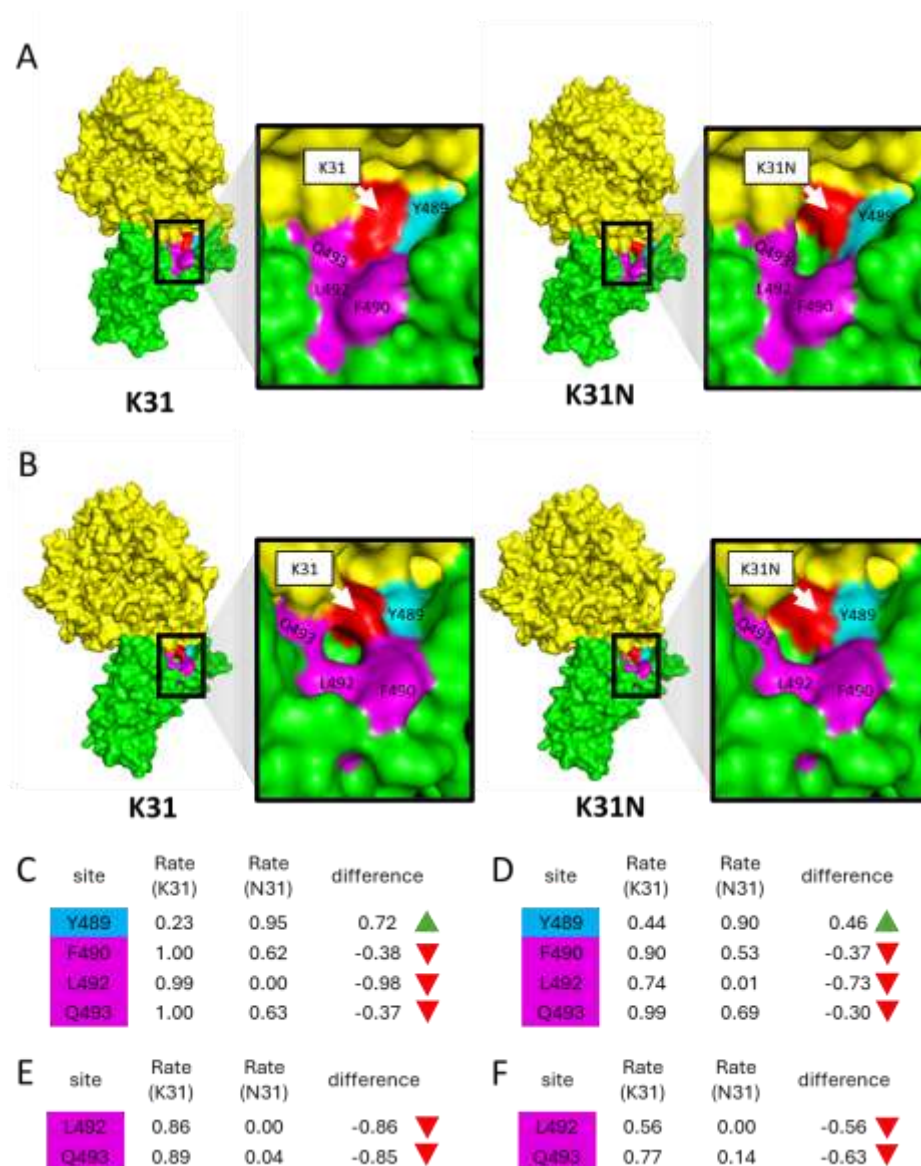


Figure 2. White-tailed deer ACE2 (marked as K31) in complex with prototype SARS-CoV-2 spike protein receptor-binding domain (RBD) (PDB ID:8HG0) (A) and Omicron RBD (PDB ID:8IFZ) (B). ACE2 is shown in yellow and RBD in green. WTD ACE2 (K31) with introduced K31N mutation to mimic sika deer ACE2 structure is marked as K31N. K31 and K31N are highlighted in red. The key amino acids in the SARS-CoV-2 RBD that are important for binding with K31 and K31N are marked with one-letter codes and colored. Sites where interaction with the K31N mutation creates weaker binding are highlighted in magenta, and those where binding increases - in blue (A-D). K31 and K31N van der Waals (C, D) and hydrogen bond (E, F) interaction rates with RBD predicted with molecular dynamics simulations for prototype (C, E) and Omicron (D, F) are shown. A positive value in 'difference' indicates decreased interaction with K31N mutation (red arrows) and negative – increased (green arrow).

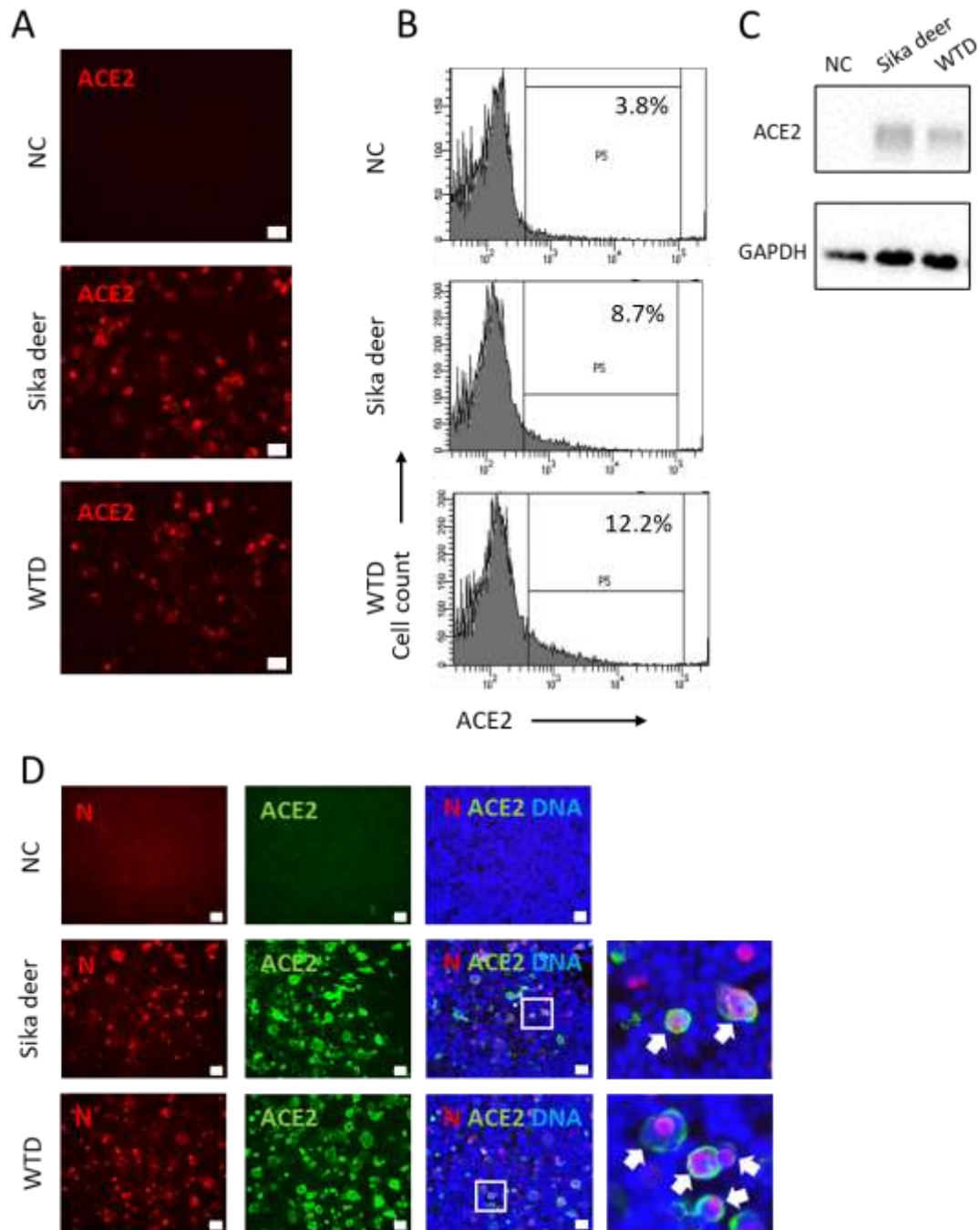


Figure 3. ACE2 expression in HEK293T cells and cell susceptibility to SARS-CoV-2 infection. Cells were transfected with plasmids encoding sika deer or white-tailed deer (WTD) ACE2, and transient ACE2 expression was confirmed at 1 day post-transfection (ACE2; red). A) Cells were fixed and stained with mouse monoclonal anti-ACE2 antibodies and secondary anti-mouse IgG conjugated with Alexa Fluor 594 (red) for immunofluorescent microscopy. Scale bar = 50  $\mu$ m. NC, negative control, non-transfected cells. B) Cells were detached and stained with mouse monoclonal anti-ACE2 antibodies and secondary anti-

571 mouse IgG conjugated with Alexa Fluor 488 for flow cytometry. Histograms show cell counts of each  
572 fluorescent signal intensity. The ratio of cells expressing ACE2 per total cells is indicated in the top-right  
573 corner of each graph. C) Cell lysates were subjected to western blotting. Staining was performed with  
574 mouse anti-ACE2 monoclonal antibody and anti-GAPDH monoclonal antibody. D) HEK293T cells  
575 transiently expressing sika deer and WTD ACE2 were infected with SARS-COV-2 WK-521 strain,  
576 followed by an immunofluorescent assay at 24-h post-infection. Anti-SARS-CoV-2 N protein (N; red),  
577 anti-ACE2 (ACE2; green) antibodies, and DAPI (DNA; blue) were used for staining. White arrows indicate  
578 infected cells expressing ACE2. NC, negative control HEK293T cells. Scale bar = 50  $\mu$ m. White squares  
579 were magnified on the right of the images.

Fig. 5

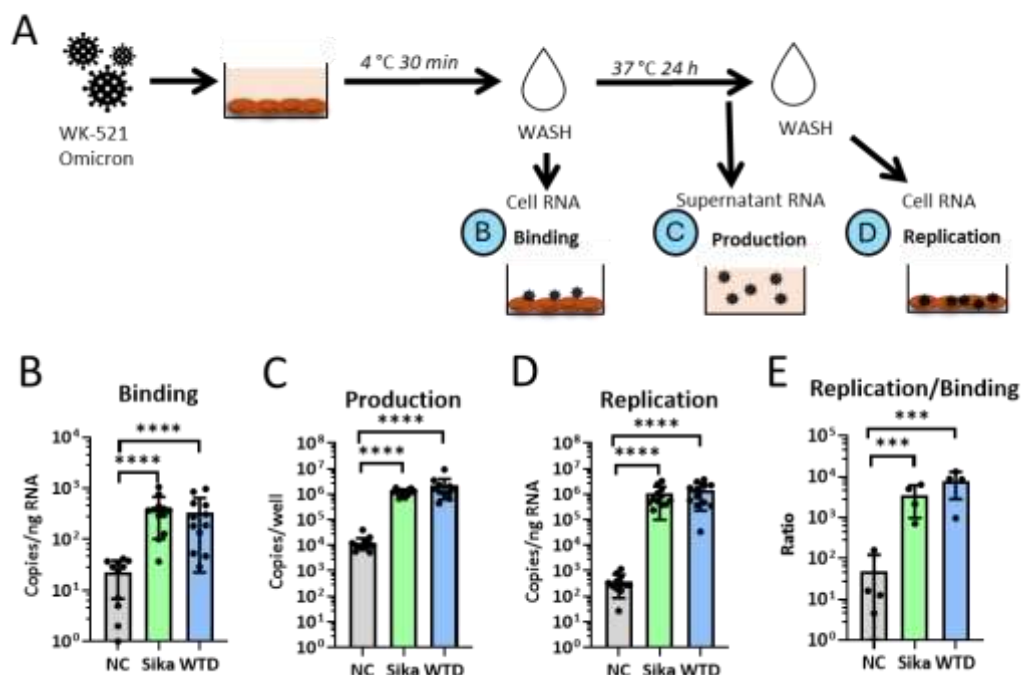
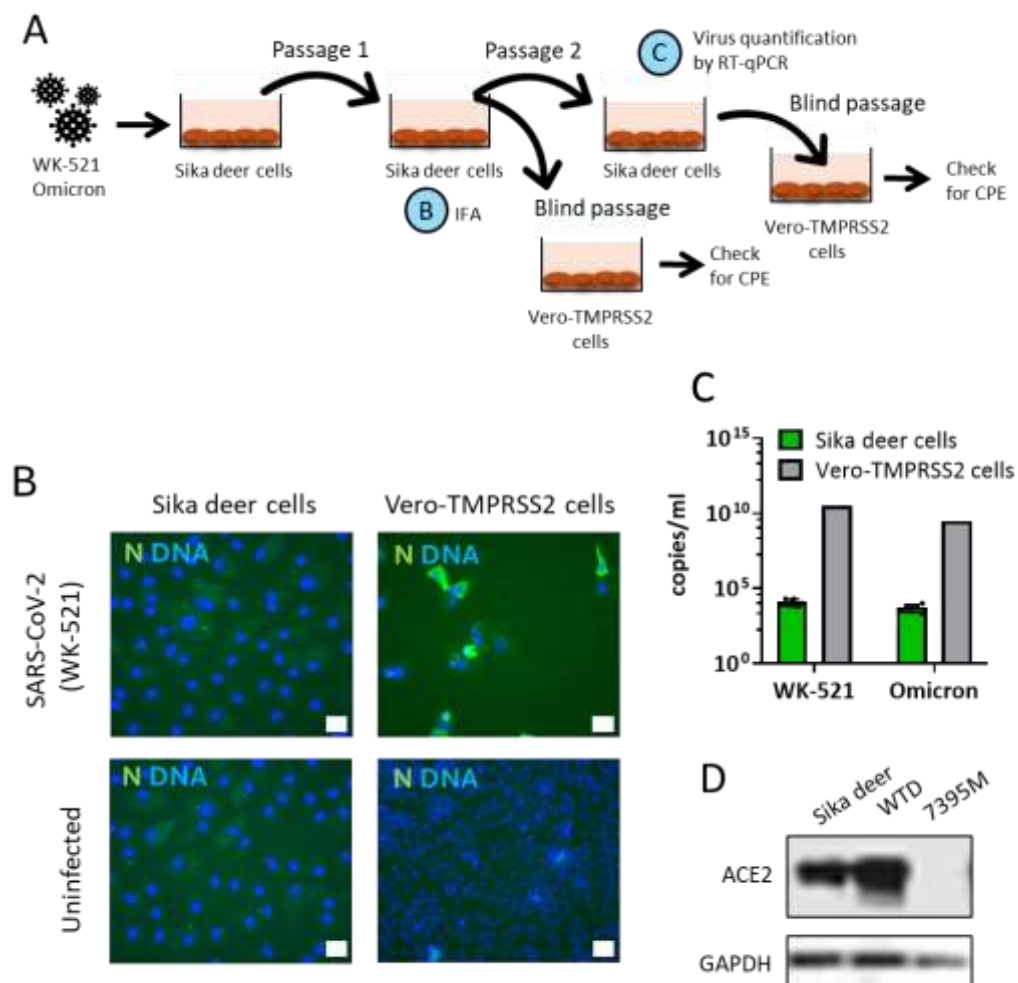


Figure 4. Virus binding and cell entry in sika deer and white-tailed deer (WTD) ACE2-expressing HEK293T cells. A) Schematic representation of the assay to quantify the susceptibility of each cell to SARS-CoV-2 is shown. The amount of viruses was measured for each step of virus infection, i.e., binding (B), production (C), and replication (D), by RT-qPCR assay. Y-axis indicates SARS-CoV-2 RNA copies per total cell RNA (B, D) or per well (C). Each dot represents the RT-qPCR value from a single well in four independent experiments, performed in duplicate or triplicate. Group means with standard deviation (SD) are shown. E) The ratio of viral RNA in the replication assay to that in the binding assay was calculated based on the mean values from each of the four independent experiments. Error bars represent the geometric mean with SD. NC: negative control HEK293T. \*\*\*\*:  $p < 0.001$ , \*\*\*:  $p < 0.01$ , \*\*:  $p < 0.05$ , and  $p > 0.05$ : not shown, by one-way ANOVA with Tukey's multiple comparisons of the means test.



Supplementary Figure 1. Virus replication assessment in sika-deer primary cells. A) Schematic representation of SARS-CoV-2 infection study using sika-deer primary cells is shown. Vero-TMPRSS2 cells were used as a positive control. Steps at which cells were stained for IFA in (B) and supernatant collected for RT-qPCR (C) are shown. B) Representative figures of the primary sika deer cells inoculated with the SARS-CoV-2 WK-521 strain. Cells were stained with antibodies targeting SARS-CoV-2 N protein (N), and nuclei were counterstained with DAPI (DNA). Vero-TMPRSS2 cells infected at 0.0002 MOI and fixed on 3dpi were used as a positive control. White scale = 50  $\mu$ m. C) Cell supernatant of six sika deer primary cell lines collected on 8 dpi in passage 2. Mean values of virus copy number as measured by RT-qPCR for each cell line (dots) and mean with SD are shown. Supernatant of the 3dpi Vero-TMPRSS2 cell culture was used as a control for comparison. D) Western blotting results for sika deer and WTD ACE2-expressing cells, and sika deer primary cells (7395M). Staining was performed with a mouse anti-ACE2 monoclonal antibody and anti-GAPDH monoclonal antibody.

609    **List of Abbreviations:**

610    ACE2: angiotensin-converting enzyme 2

611    Bp: Base pair

612    CLP: Collectrin-like part

613    COVID-19: Coronavirus diseases 2019

614    CPE: cytopathic effect

615    DAPI: 4',6-Diamidino-2-Phenylindole Dihydrochloride

616    DMEM: Dulbecco's modified minimal essential medium

617    dpi.: day post infection

618    dpt: day post transfection

619    ELISA: Enzyme-Linked Immunosorbent Assay

620    FACS: Fluorescence-activated cell sorting

621    FBCS: fetal bovine serum

622    IFA: immunofluorescence assay

623    JIHS: Japan Institute for Health Security

624    MD: Molecular dynamics

625    MEGA: Molecular Evolutionary Genetics Analysis

626    MOI: multiplicity of infection

627    NCBI: National Center for Biotechnology Information

628    NIES: National Institute of Environmental Studies

629    NIID: National Institute of Infectious Diseases

630    PBS: phosphate buffered saline

631    PD: Peptidase domain

632    PDB: Protein Data Bank

633    PFA: paraformaldehyde

634    PVDF: polyvinylidene difluoride

635    RT-qPCR: Real-time RT-PCR

636    SARS-CoV: severe acute respiratory syndrome coronavirus

637    TBS-T: Tris Buffered Saline with Tween 20

638    TCID<sub>50</sub>: median tissue culture infectious dose

639    VNA: neutralization assay

640    WTD: White-tailed deer

641