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**Characterization of a mammalian orthoreovirus isolated
from the large flying fox, *Pteropus vampyrus* in Indonesia**

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Abstract

Fruit bats serve as an important reservoir for many zoonotic pathogens, including Nipah virus, Hendra virus, Marburg virus and Lyssavirus. To gain a deeper insight into the virological characteristics, pathogenicity and zoonotic potential of bat-borne viruses, recovery of infectious viruses from field samples is important. Here, we report the isolation and characterization of a mammalian orthoreovirus (MRV) from a large flying fox (*Pteropus vampyrus*: *P. vampyrus*) in Indonesia, which is the first detection of MRV in Southeast Asia. MRV was recovered from faecal samples of three different *P. vampyrus* in Central Java. Nucleotide sequence analysis revealed that the genome of the three MRV isolates shared more than 99% nucleotide sequence identity. We tentatively named one isolated strain as MRV12-52 for further analysis and characterization. Among 10 genome segments, MRV12-52 S1 and S4 which encode the cell-attachment protein and outer capsid protein, had 93.6 and 95.1% nucleotide sequence identities with known MRV strains, respectively. Meanwhile, the remaining genome segments of MRV12-52 were divergent with 72.9-80.7% nucleotide sequence identities. Based on the nucleotide sequence of the S1 segment, MRV12-52 was grouped into serotype 2, and phylogenetic analysis demonstrated evidence of past reassortment events. *In vitro* characterization of MRV12-52 showed that the virus efficiently replicated in BHK-21, HEK293T and A549 cells. In addition, experimental infection of laboratory mice with MRV12-52 caused severe pneumonia with 75% mortality. This study highlights the presence of pathogenic MRV in Indonesia, and which could serve as a potential animal and public health concern.

Keywords: Virus isolation; Viral pathogenicity; Mammalian orthoreovirus; Fruit bats; Indonesia

Introduction

The outbreak of diseases originating from bats including severe acute respiratory syndrome coronavirus (SARS-CoV), SARS-CoV-2, Middle East respiratory syndrome coronavirus (MERS-CoV), Marburg and Nipah viruses has encouraged the search to identify new and novel viruses from bats that could potentially cause an epidemic or even a pandemic. Although numerous viral sequences have been identified from various species of bats worldwide; isolation of infectious viruses have been relatively unsuccessful. Genetic information of viruses detected from bats important for tracking their origins, evolution and geographical distribution, but not enough to evaluate their molecular and pathogenic properties. Thus, viral isolation from field samples remains essential to gain a deeper insight into the virological characteristics, and the zoonotic potential of bat-borne viruses [1].

Fruit bats also serve as reservoirs of the viruses belonging to the genus *Orthoreovirus* (ORV), including Mammalian orthoreovirus (MRV), Nelson Bay Orthoreovirus (NBV) and Broome orthoreovirus (BrV) [2–12]. MRVs and NBVs are zoonotic viruses associated with gastrointestinal and respiratory tract diseases in humans; however, there is no evidence of any disease associated with BrV [12–19]. MRV was the first identified species of ORV and has emerged in a broad range of mammalian hosts, including humans, livestock, bats and wildlife [2, 17, 18, 20–29]. Moreover, it can persist in the environment and is frequently detected from sewage [30, 31]. MRV is a non-enveloped, wheel-like virion belonging to the subfamily *Spinareovirinae*, family *Reoviridae*. This contains 10 segments of double-stranded RNA which can be divided into 3 large segments (L1-L3), 3 medium segments (M1-M3) and 4 small segments (S1-S4). Based on neutralization and hemagglutination activities of the cell attachment protein encoded by the S1 segment, MRVs can be classified into 4 serotypes: Type 1 Lang, Type 2 Jones, Type 3 Dearing and Type 4 Ndelle [32–34].

MRVs have been identified and isolated from various species of insectivorous bats in China, Japan, South Korea, Slovenia, Germany, Italy, Canada and the United States of America (USA) [24–26, 29, 35–40]. Bat MRVs displayed diverse genetic backgrounds and are classified into serotypes 1-3 [8, 24, 26, 29, 35, 38–41]. Genomic reassortment events occur in segmented viruses and are important in viral evolution and the emergence of virus reassortment strains. Most bat MRV genomes are related to those detected in humans, pigs and wildlife, suggesting cross-species transmission of the virus among these hosts, and is the basis for reassortment of genome segments with the other MRV strains [25, 26, 29, 35, 38, 39, 42]. Although many bat MRVs are closely related to human MRVs, evidence for spillover from bats to humans is still limited and needs to be further elucidated.

There have been numerous reports on the identification of herpesviruses, parvoviruses, polyomaviruses, paramyxoviruses, coronaviruses and NBV from Indonesian fruit bats [11, 43–48]. Among these viruses, infectious herpesvirus and NBV were recovered from tissues and faeces of the bats [11, 48]. In the present study, we have conducted a virus isolation-based approach to identify infectious viruses and successfully isolated MRV from fecal samples of the large flying fox (*Pteropus vampyrus*) collected in Central Java, Indonesia. To characterize the genetic and virological properties of a representative isolate (MRV12-52), we then determined the whole genome sequence of MRV12-52 and examined growth efficiency in cell lines and the pathogenicity of MRV12-52 in laboratory mice.

Materials and Methods

Bat sampling

Bat sampling was performed in Sumatra (Lima Puluh Kota), Java (Panjalu, Magelang and Surabaya) and Sulawesi (Popayato, Paguyaman, Sidrap and Soppeng), in Indonesia from 2010 to 2014. Tissue samples were collected from bats euthanized with an overdose of ketamine and xylazine by veterinarians wearing personal protective equipment and were exported under the permission of the Directorate General of Livestock and Animal Health Services, Ministry of Agriculture, Republic of Indonesia. All samples were kept at -80°C and used for virus screening as described previously [46].

Cells and viruses

Vero E6, Vero-expressing human TMPRSS2 (Vero T2), Vero T2-co-expressing human ACE2 (Vero T2-hACE2), Vero T2-co-expressing bat ACE2 and A549 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Caco-2 cells were grown in DMEM supplemented with 1% non-essential amino acid and 10% FBS in a collagen-coated plate, and HEK293T cells were cultured in high glucose DMEM with 10% FBS. BHK-21 cells were grown and maintained in Minimum Essential Medium (MEM) supplemented with 10%FBS. Vero T2-expressing *Acerodon celebensis* ACE2 (Vero T2-AcACE2) and Vero T2-expressing *Dobsonia moluccensis* ACE2 (Vero T2-DmACE2) that were generated for virus isolation were cultured in the same condition as Vero E6. All cells were cultured at 37°C with 5% CO₂.

The isolated viruses (MRV12-47, MRV12-48 and MRV12-52) were propagated in Vero T2-hACE2 or BHK-21 and the virus stocks were titrated by 50% tissue culture infectious dose (TCID₅₀) or focus forming assay (FFA) using BHK-21 cells.

Virus isolation

Faecal and lung samples of fruit bats were suspended in phosphate buffer saline at 10% w/v and homogenized using beads as described previously [11]. Throat swab samples were collected and kept in 1 ml of viral transport medium. The samples were centrifuged at 6,000 x g for 3 min and supernatants were filtered using Vivaclear mini clarifying filter 0.8 µm (Sartorius, Stonehouse, UK). Virus isolation was conducted in the biosafety level 3 facility at International Institute for Zoonosis Control, Hokkaido University. In brief, Vero T2-AcACE2, Vero T2-DmACE2 and Vero T2-hACE2 cultured in 12 well plates were inoculated with 20 µl of the suspensions. Cells were maintained in DMEM supplemented with 2% FBS, 2% antibiotic-antimycotic solution (Wako, Osaka, Japan) and gentamicin (25 µg/ml). Blind passage was performed twice after 7 days of culture. Cells were monitored daily for the appearance of cytopathic effects (CPEs) and the supernatants from cells showing CPEs was collected and kept at -80°C as master stocks of the isolated viruses.

Identification of virus morphology by electron microscopy

The isolated viruses were propagated in Vero T2-hACE2 cells and pelleted by ultracentrifugation at 110,800 x g for 1.5 h with a 20% sucrose cushion and resuspended in PBS. The virus particles were fixed with 4% paraformaldehyde, loaded onto polyvinyl-coated nickel grids (Nissin EM, Tokyo, Japan) and negatively stained with 2% phosphotungstic acid solution (pH 5.8). The morphology of the isolated viruses was observed under a transmission electron microscope Hitachi HT7800 TEM (Hitachi Ltd., Ibaraki, Japan).

High-throughput sequencing

Total RNA was extracted from the culture supernatant of cells infected with MRV12-47, MRV12-48 and MRV12-52 with a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) and reverse transcribed into a double-strand cDNA using PrimeScript Double Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The sequence library was constructed using Nextera XT DNA Library Preparation Kit and sequenced on an Illumina MiSeq instrument (Illumina, San Diego, USA).

Sequence and phylogenetic analysis

The contigs of viral genome segments were recovered from high-throughput sequence reads by *de novo* assembly and mapped to reads approach using CLC Genomics Workbench 20.1 (Qiagen, Venlo, Netherlands). For phylogenetic analysis, coding nucleotide sequences of the reference MRVs were obtained from the GenBank database. The reference sequences were aligned with MRV12-47, MRV12-48 and MRV12-52 sequences using MUSCLE alignment. General Time Reversible (GTR) with gamma-distributed with invariant sites (G+I) was used as a mathematical model for construction of maximum-likelihood tree of L1-L3, M1, M2 and S3, while the Tamura-Nei model with G+I was used to construct the phylogenetic tree of M3, GTR+G was used for the S1 and S2 trees, and Kimura 2 with G was used for the S4 tree. The algorithm was analyzed with a bootstrapping value of 1000 replicates. The trees were visualized using the Interactive Tree of Life (iTOL) version 6.8.1 [49].

Production of anti-MRV polyclonal antibody

Five-week-old female Hartley guinea pigs (Japan SLC Inc., Shizuoka, Japan) were intranasally inoculated with 2.5×10^6 TCID₅₀ of MRV12-52. The animals were boosted twice with the same dose *via* intraperitoneal injection at 2 and 4 weeks after the first immunization.

At 42 days post infection (dpi), the animals were euthanized by excessive isoflurane inhalation and blood was collected via cardiac puncture. After heat-inactivation at 56°C for 30 min, the sera were stored at -80°C and used as anti-MRV12-52 polyclonal antibody for virus titration by FFA, immunofluorescence assays (IFAs) and immunohistochemistry (IHC).

Virus titration by FFA

The specimens were 10-fold serially diluted and inoculated onto monolayers of BHK-21 cells cultured in 24 well plates. The plates were incubated at 37°C for 1 h and then cultured in DMEM containing 2% FBS and 0.8% methylcellulose. Cells were fixed with 10% buffered formalin at 36 hours post infection (hpi). Cells were blocked with 50% Block Ace (KAC, Kyoto, Japan) in PBS and then stained with 1:3,000 of guinea pig anti-MRV12-52 antibody and Alexa-Fluor 488-conjugated goat anti-guinea pig IgG (Invitrogen; Thermo Scientific, Waltham, USA) as a secondary antibody. Cell nuclei were stained with Hoescht 33342 (Invitrogen). Viral antigen-positive foci were counted under a fluorescence microscope (IX73, Olympus, Tokyo, Japan). Virus titers were calculated as focus-forming units per milliliter (ffu/ml) based on the number of foci.

Growth kinetics and immunofluorescence staining of MRV12-52 in cell lines

Vero E6, Vero T2, Vero T2-hACE2, BHK-21, A549, Caco-2 and HEK293T cells cultured in 12 well-plates were inoculated with MRV12-52 at a multiplicity of infection (MOI) of 0.01. Triplicate of cell culture supernatants were collected from each cell line at 0, 24, 48 and 72 hpi and viral titers were examined by FFA as described above.

For IFA, cells were inoculated with MRV12-52 at an MOI of 0.01 and cultured in maintenance media supplemented with 2% FBS. Cells were fixed at 48 hpi and stained with

the same protocol as FFA. The merged images were prepared using CellSens Imaging Software (Olympus).

Animal experiments

Four-week-old, female BALB/c mice (Japan SLC Inc.) were intranasally inoculated with 10^6 ffu of MRV12-52 in 50 μ l PBS or PBS only as mock infection. For survival analysis (control; n=3, MRV12-52 inoculation; n=8), the body weight was monitored daily up to 14 dpi. The humane endpoint was established as body weight loss exceeding 20%. For virus quantification, mice (n=5) were sacrificed at 1, 3, 5 and 7 dpi, and the organs, including lung, liver, spleen, kidney and intestine were harvested in 3 ml of PBS. The lungs were collected in 10% buffered formalin from 3 mice (n=3) at 3, 5 and 7 dpi for histopathological examination.

Virus quantification in the organs of mice

The organs from mice were homogenized in PBS using a TissueRuptor (Qiagen). To examine viral RNA levels, total RNA was extracted from homogenized lung, liver, spleen, kidney and intestine with TRIzol LS reagent (Invitrogen) and Direct-Zol RNA MiniPrep kit (Zymo Research, Irvine, USA). Blood was collected into EDTA tube by cardiac puncture and subjected to RNA extraction using a High Pure Viral Nucleic Acid kit (Roche Diagnostics). Quantitative RT-PCR (qRT-PCR) assay was performed with THUNDERBIRD Probe One-step qRT-PCR Kit (TOYOBO, Osaka, Japan). Primer and probe sequences for MRV12-52 L2 segment were CCCGCGACTCCTGTATTATTC-3', 5'-CACTGCCGACGATACTTCATAG-3' and 5'-FAM-CGTCGCCTC-ZEN-ATGTGTGTC-IBFQ-3'. The expression levels of endogenous mouse beta-actin were quantified for normalization with Taqman mouse beta-actin Endogenous Control (Mm00607939_s1, Applied Biosystems; Thermo Scientific). The relative gene expression of the L2 segment was calculated by $2^{-\Delta C_t}$ method.

To determine viral titers in organs, homogenized tissues were cleared by centrifugation and titrated by FFA as described above.

Histopathological examination

The fixed lungs were processed for paraffin embedding and sectioned at 3 μ m. The lung sections were deparaffinized and stained with Hematoxylin and Eosin (H&E) for histopathological examination. For IHC, the sections were deparaffinized and heated in citrate buffer for 3 min using a pressure cooker for antigen retrieval. The sections were stained with guinea pig anti-MRV12-52 polyclonal antibody (developed in this study), anti-CD3- ϵ (sc-1127, Santa Cruz Biotechnology, Dallas, USA), anti-Iba1 (019-19741, Fujifilm-Wako, Osaka, Japan) or anti-myeloperoxidase (MPO) (A039829-2; Dako; Agilent, Santa Clara, USA). Immunostaining was detected with the EnVision system peroxidase-labeled anti-rabbit immunoglobulin (Dako; Agilent) or VECTASTAIN ABC guinea pig IgG Kit (Vector Laboratories, USA) and then visualized using a Histofine diaminobenzidine substrate kit (Nichirei Biosciences, Tokyo, Japan).

Nested RT-PCR screen of MRV genome in fecae samples

Total RNA was extracted from faecal samples of fruit bats (n=91) as described in the previous study [11] and examined for MRV genome by nested RT-PCR. Two primer sets were designed targeting S4 segment of the isolated MRV. The first round RT-PCR was conducted with PrimeScript II High fidelity RT-PCR kit (Takara Bio) using primers 55F; 5'-AACGCTTTTGAAGGTCGTGTGTC-3' and 1029R; 5'-GAGAGCAGCAGGAGTCAGTACGATTG-3'. The second round PCR was performed with KOD One PCR Master Mix (TOYOBO) using 195F primer; 5'-ACGCAAGCTGAAACATTTGCCTCATC-3' and 737R primer; 5'-ACCAACTCCTTTCTATATGCACGGCC-3'. PCR product with approximately

543 bp in length was sequenced by Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Scientific).

Statistical analysis and reproducibility

Data were analyzed and presented as the mean with standard deviation (SD). Statistical analysis was performed using Prism 8 (GraphPad Software, San Diego, CA). Survival analysis was performed using the Kaplan-Meier estimator. One-way ANOVA with Dunnett's test was used to determine the statistical significance of gene expression in the mouse lungs. Two-way ANOVA with Turkey's multiple comparisons was used to compare statistical differences between viral titers in Vero E6, Vero T2 and Vero T2-hACE2 cells at each time point. All experiments were repeated at least twice to ensure reproducibility.

Results

Virus isolation from Indonesian fruit bats (IFBs) and morphological identification

In this study, we initially intended to screen fruit bats collected in Indonesia for zoonotic coronaviruses using a virus isolation-based approach. Supernatants from homogenized lungs (n=167), faeces (n=91), and throat swabs (n=45) were inoculated into Vero T2-AcACE2, Vero T2-DmACE2, and Vero T2-hACE2 cells. After a second blind passage, CPEs with cell rounding and detachment were observed in Vero T2-hACE2 cells inoculated with faecal samples ID: IFB12-47, IFB12-48 and IFB12-52 that were collected from *Pteropus vampyrus* (*P. vampyrus*) in Magelang, Central Java in 2012 (Figure 1A and Table 1). We examined the morphology of the isolated virus by transmission electron microscope (TEM). Virus particles were approximately 80 nm in diameter, had a spherical wheel-like shape, and exhibited a double-layered capsids, which are the typical characteristics of reovirus (Figure 1B).

Genetic characterization and phylogenetic analysis of the isolated viruses

We performed whole genome sequencing using next-generation high-throughput sequencing. According to BLAST searches, the nucleotide sequences of the isolated viruses were highly similar to those of MRVs. Complete genome sequences consisting of the coding region and the termini of 10 genome segments were obtained from the culture supernatants of Vero T2-hACE2 cells inoculated with faeces of IFB12-47, IFB12-48, and IFB12-52. We designated the three isolated viruses as MRV12-47, MRV12-48, and MRV12-52. Nucleotide sequences of the 10 segments were deposited in the DNA Data Bank of Japan (DDBJ) with accession numbers LC773568-LC773597 (Supplemental Table S1). A pairwise comparison of each genome segment of the three isolates showed more than 99% identity (Supplemental Table S2), indicating that all isolates are the same virus strain. In this study, we selected MRV12-52 as a representative isolate for further characterization. The sizes of each genome segment of MRV12-52 estimated by the result of high-throughput sequencing were L1, 3,854 bp; L2, 3,915 bp; L3, 3,901 bp; M1, 2,304 bp; M2, 2,203 bp; M3, 2,241 bp; S1, 1,437 bp; S2, 1,331 bp; S3, 1,198 bp and S4, 1,197 bp. A pairwise comparison of coding sequence of 10 genome segments of MRV12-52 to the other known MRV strains demonstrated diverse nucleotide sequence identities ranging from 72.9 to 95.1% (Table 2). Gene encoding core structures including L1-L3, M1-M3, S2 and S3 had low nucleotide sequence identities (72.9% to 80.7%) to MRV strains isolated from pig, bat, human, and mink (Table 2). In contrast, the gene encoding the cell attachment protein (S1) was highly similar to MRV serotype 2 strain OV204 which was isolated from deer in the USA (93.6% identity) [42]. The gene encoding the major outer-capsid protein (S4) showed 95.1% nucleotide sequence identity with Osaka 1994 strain, which was isolated from an infant with meningitis in Japan [20].

L-class, M-class, S2 and S3 segments of the isolated MRVs formed distinct lineages from the other MRVs (Figures 2 and 3), while S1 and S4 segments were genetically close to

most of the MRV strains (Figure 3). MRVs can be classified into 4 serotypes based on the nucleotide sequence of the S1 segment. Phylogenetic analysis of the S1 segment demonstrated that MRV12-47, MRV12-48 and MRV12-52 were assigned to serotype 2 (Figure 3).

Analysis of MRV12-52 genome exhibited diverse nucleotide and amino acid sequences with the other MRV strains (Table 2). Especially, the M3 segment of MRV12-52 showed relatively low sequence identity with known MRVs. MRV M3 segment encodes the core non-structural protein μ NS. The C-terminal domain of μ NS has been reported to associate with the viral RNA-dependent RNA polymerase λ 3 and be essential for the formation of inclusion bodies termed virus factory-like structures (VFL) [50] (Figure 4A). Consistent with the sequence diversity, multiple amino acid sequence alignment of μ NS identified unique amino acid residues in μ NS of MRV12-52 (Figure 4B), suggesting the unique evolutionary background of MRV12-52.

Prevalence of MRV in IFBs

We examined the prevalence of the isolated MRV using faecal samples of IFBs by nested RT-PCR targeting viral S4 genome segment. The viral genome was detected only in 3 faecal samples of *P. vampyrus* (IFB12-47, IFB12-49, and IFB12-52), consisting of two samples positive for virus isolation (Supplemental Table S3). We note that our previous nested RT-PCR screening using pan-orthoreovirus primer sets also detected no MRV in same bat RNA samples [11]. These results imply the low prevalence of MRV in Indonesian bats, but further investigations are needed to infer the prevalence of MRV in IFBs.

Growth efficiency of MRV12-52 in cell lines

We examined the growth efficiency of MRV12-52 in Vero E6, BHK-21, A549, Caco-2 and HEK293T cells which were inoculated at an MOI=0.01, and the viral titers in culture

supernatants were quantified by FFA. In BHK-21, the titers of MRV12-52 were over 10^5 ffu/ml at 24 hpi and reached to 10^7 ffu/ml at 72 hpi (Figure 5A). HEK293T and A549 cells also supported the efficient proliferation of MRV12-52, while Vero E6 and Caco-2 cells were relatively less permissive to infection (Figure 5A). Immunofluorescence staining of MRV12-52 antigens in each cell line at 48 hpi illustrated abundant viral antigens in BHK-21, HEK293T and A549 cells (Figure 5B), supporting the results of growth curve analysis.

In this study, we isolated bat MRVs using Vero T2-hACE2 cells. To investigate the impact of TMPRSS2- and ACE2-expressions on MRV infection, we compared the growth efficiency of MRV12-52 in Vero E6, Vero T2 and Vero T2-hACE2. The titers of MRV12-52 in Vero E6, Vero T2 and Vero T2-hACE2 were not significantly different at all time points (Figure 5C), indicating that the expression of TMPRSS2 and ACE2 in Vero cells did not enhance the growth of MRV12-52.

Experimental infection of laboratory mice with MRV12-52

To investigate the infectivity and pathogenicity of MRV12-52, we examined viral loads, pathological changes and survival rates of BALB/c mice inoculated with MRV12-52 (Figure 6A). The mortality rate caused by MRV12-52 in mice was 75% (Figure 6B). The mice showed body weight decreases from 6 to 7 dpi (Figure 6C). Six out of eight (75%) mice developed severe body weight loss and reached the endpoint at 7 or 8 dpi. Two (25%) mice decreased their body weight slightly but recovered from the infection.

Next, we examined the replication of MRV12-52 in blood and primary organs, including lung, liver, spleen, kidney, and intestine of mice. The viral titer in the lungs was significantly increased at 3 dpi and reached a peak at 5 dpi, thereafter the titer slightly declined at 7 dpi (Figure 6D). The virus titer was also detected in the intestine at 1 dpi and then diminished at 3, 5 and 7 dpi. However, the titers in the liver, spleen and kidney were below the

limits of detection indicating that MRV12-52 replicated primarily in the lung and intestine (Figure 6D). Consistently, RNA of the viral L2 gene was abundant in the lung at 3 dpi and decreased at 5 and 7 dpi (Figure 6E). Viral RNA also detected in intestine from 1 dpi and gradually decrease in time-dependent manner. Low levels of viral RNA were detected in blood and other organs.

Lung pathology of MRV12-52-infected mice

To evaluate pathological changes caused by MRV12-52 infection, mice were sacrificed at 3, 5 and 7 dpi (Figure 6A). Hyperemia with edema were mainly observed at the right middle and inferior lobes of the lung of mice at 3 dpi. Lung pathology was shown to progress at 5 dpi and became more severe at 7 dpi with edema throughout the lungs (Figure 7A). Microscopic examination of the lung sections at 3, 5 and 7 dpi demonstrated diffuse inflammatory cell infiltration in the peribronchovascular area and alveolar space. Lymphocytic infiltration, interstitial pneumonia, and alveolar thickening were found in the lung at 5 and 7 dpi (Figure 7B), concordantly with the gross appearance of MRV12-52 infected lungs (Figure 7A). To detect viral antigens by IHC, we prepared anti-MRV12-52 polyclonal antibody by immunizing guinea pigs with MRV12-52. Our IHC demonstrated that MRV12-52 antigen was found abundantly in alveolar epithelial cells at 3 and 5 dpi but was limited at 7 dpi (Figure 7C). Furthermore, we characterized inflammatory cells at 7 dpi by IHC with anti-CD3 antibody for T lymphocytes, anti-Iba1 antibody for macrophages and anti-MPO antibody for neutrophils. Iba1-positive macrophages were the major immune cells seen in the responses to MRV12-52 infection. T lymphocytes were also found diffusely distributed in the alveolar space, while neutrophils were rarely detected (Figure 7D).

Discussion

The emergence of bat ORVs has become a public health concern in the past few decades. Several bat ORVs belonging to NBV species, including Kampar, Melaka, Sikamat, Miyazaki-Bali/2007, HK23629/07, HK46886/09 and HK50842/10 have been reported to cause acute respiratory tract infections in humans, especially in Malaysia and Indonesia, indicating the emergence of ORV spillover from bats to humans in Southeast Asia (SEA) region [13–16, 19]. Previously, we also have isolated NBV, named Paguyaman orthoreovirus (PgORV) from fruit bats in Indonesia [11]. Other ORV species MRV, have been widely reported from livestock, bats, wildlife, humans and environmental samples in many countries and regions, including China, Japan, Korea, Taiwan, Europe, Africa and America [2, 17, 18, 20–31]. However, there has been no reports on MRV in bats, wildlife, livestock or humans in the SEA region, while NBV has been detected sporadically in fruit bats and humans in several SEA countries including Malaysia, Indonesia and the Philippines. In the current study, we recovered 3 MRV isolates, MRV12-47, MRV12-48 and MRV12-52 from IFBs. This study is the first report on the isolation and characterization of MRV in the SEA region.

Fruit bats belonging to the family *Pteropodidae* serve as natural reservoirs of NBV [5–11, 51] In contrast, most MRVs have been identified and isolated from various species of insectivorous bats including, *Rhinolophus spp.*, *Myotis spp.*, *Hipposideros spp.*, *Pipistrellus spp.*, *Vespertilio spp.*, *Eptesicus spp.*, *Lasiurus spp.* and *Lasionycteris spp.*, suggesting that insectivorous bats may play an important role as a main reservoir of bat MRVs [7, 24–26, 29, 35, 36, 38–40]. In our study, we detected and recovered MRVs from faecal samples of large flying foxes (*P. vampyrus*). Moreover, another study has reported MRV isolated from short-nosed fruit bats (*Cynopterus sphinx*) in China [2]. These findings indicated that fruit bats as well as insectivorous bats can harbor MRVs.

Genes encoding core proteins, including L1-L3, M1, M3, S2 and S3 were highly conserved among MRVs with more than 85% of nucleotide sequence identity [21, 22, 24–26,

30, 35]. Conversely, pairwise comparison of L1-L3, M1-M3, S2 and S3 segments of MRV12-52 displayed low nucleotide sequence identities ranging from 73.3 to 80.8% to the closest MRV strains. Moreover, phylogenetic analysis of these segments displayed the unique lineage of MRV12-52 that was evolutionary distinct from the known MRV strains. Multiple sequence alignment of the representative core protein, μ NS showed significant amino acid sequence differences between MRV12-52 and the prototype MRV strains at positions 471 to 721, where is the domain associated with VFL formation in MRV-infected cells. These data highlighted the unique genetic background of MRV12-52, the first MRV isolated from fruit bats in Indonesia. Because of MRVs have high diversity in their genome segments, the recent study on MRV genome constellations proposed that the genotypes of MRVs can be assigned to each segment and the minimum intragenotype sequence identities of each segment range from 77-88% [52]. From our data, L2, M1 and M3 segments displayed sequence identities of 75.7, 73.3 and 74.8%, respectively, suggesting that these segments of MRV12-52 may form unique genotypes from the other MRV strains. However, unfortunately these data are not sufficient to conclude whether such unique genome characteristics will be found in the other animals and humans in SEA. Continuing studies of genetic evolutions in bats, wildlife, livestock and humans will bridge the gap of knowledge and provide more information on the circulation and distribution of MRV in the SEA region.

Reassortments were evident in several MRV strains [24, 25, 42, 53, 54]. In the present study, S1 and S4 segments of MRV12-47, MRV12-48 and MRV12-52 had high sequence identities with those of MRVs from deer (93.6%) and humans (95.3%), respectively. Phylogenetic analysis revealed that MRV12-47, MRV12-48 and MRV12-52 formed a cluster with known MRVs in the trees of the S1 and S4 segments but were segregated in the trees of L1, L2, L3, M1, M2, M3, S2 and S3 segments. These incongruences suggest past reassortment event(s) between the MRV12-47, MRV12-48 and MRV12-52 genomes and the other strains

identified from deer and humans, implying cross-species transmission in bats, wildlife and humans.

MRVs cause a variety of diseases depending on the host and virus strains. MRV serotype 3 has been reported to have high pathogenicity in pigs causing diarrhea and respiratory distress that affects animal production with subsequent economic loss [22, 23, 53, 55]. In humans, MRV infection results in asymptomatic or mild acute gastroenteritis [17, 18]. Occasionally, MRV can cause meningitis and encephalitis in children [20, 56, 57]. Although several MRV strains were identified in bats, there is little evidence for virus spillover from bats to humans. Only one MRV strain (SI-MRV01) which was highly similar to the MRV strain isolated from a bat in Germany (342/08) has been reported from an infant with gastroenteritis in Slovenia [17].

At the beginning of this study, our virus isolation screening targeted sarbecoviruses and related viruses in IFBs. It has been reported that sarbecoviruses were successfully isolated from *Rhinolophus cornutus* using Vero cells stably expressing human TMPRSS2 and *R. cornutus* ACE2 (Vero-RcACE2 cells) [58]. These bat sarbecoviruses were not able to utilize human ACE2 as a receptor indicating the narrow host range [58]. Following the previous study, we established Vero cells co-expressing human TMPRSS2 and bat ACE2 (Vero T2-AcACE2 and Vero T2-DmACE2). Unfortunately, no coronaviruses were isolated in this study.

In the present study, we evaluated the properties and pathogenicity of MRV12-52 by inoculating the virus into BALB/c mice. Our result demonstrated that MRV12-52 replicated efficiently in the lung and caused severe respiratory diseases in laboratory mice with a high mortality of up to 75%. Other studies by Jiang and Li et al. also showed that intranasal inoculation of mice with MRVs isolated from bats resulted in body weight loss and respiratory distress; however, the mortality rates varied from 0-50% depending on the virus strains and

infectious doses [2, 59]. The respiratory tract and gastrointestinal tract are the main targets of MRV12-52 replication. The virus did not disseminate to other organs such as the liver, spleen or kidney. In contrast, WIV2, WIV3 and WIV7 strains isolated from insectivorous bats and the B/03 strain isolated from wild short-nosed fruit bats in China exhibited systemic infection involving the intestine, liver, spleen, brain, heart and kidney [2, 58], supporting the view that the infectivity and pathogenicity of MRV may depend on the virus strain. To assess the burden and zoonotic potential of the isolated MRVs, further epidemiological and virological studies should be performed in bat populations and humans in Indonesia.

In conclusion, we have isolated MRVs from large flying foxes in Indonesia and this is the first report of MRV in the SEA region. Characterization of the isolated MRV (MRV12-52) suggested a unique evolutionary history and the possible previous reassortment of S1 and S4 segments with other MRVs. We also demonstrated that MRV12-52 causes lethal infection with severe pneumonia in laboratory mice. In future studies knowledge on virus characteristics, infectivity and pathogenicity will highlight the importance of MRV as a potential public health threat. Virus surveillance in bats, wildlife, livestock and humans are now needed be continued to better understand the evolution, distribution, transmission and the zoonotic potential of bat MRV in Indonesia and the SEA region.

Declaration of interest statement

The authors declare no competing interests.

Ethical approval

Bat sampling was performed under permit number 05-2010 RSHP-IPB approved by the Animal Care and Use Committee of Veterinary Teaching Hospital, Bogor Agricultural

University. Animal experiments with MRV had ethical approval from the Institutional Animal Care and Use Committee of Hokkaido University (approval number 20-0026).

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Figure legends

Figure 1. Cytopathic effects (CPEs) and virion morphology of mammalian

orthoreovirus isolated from Indonesian fruit bats (IFBs). (A) Vero T2-hACE2 cells were

inoculated with mock (left panel) or a faecal suspension of IFB12-47. Scale bar = 100 μ m.

(B) Negatively stained electron micrograph of the isolated virus by transmission electron

microscope. Scale bar = 100 nm.

Figure 2. Phylogenetic analysis of large (L) and medium (M) segments of the isolated

virus genomes. Coding nucleotide sequences of L1, L2, L3, M1, M2 and M3 segments of the

reference mammalian orthoreoviruses (MRVs), MRV12-47, MRV12-48 and MRV12-52

were aligned. Phylogenetic trees of each segment were constructed using the maximum

likelihood method with bootstrap values of 1,000 replicates. The bootstrap values above 70

were indicated in each branch. The scale bars represent the number of nucleotide

substitutions per site.

Figure 3. Phylogenetic tree of small (S) segments of the isolated viruses. Coding nucleotide

sequences of S1, S2, S3 and S4 segments of MRV12-47, MRV12-48 and MRV12-52 were

aligned with the reference MRVs. Phylogenetic trees of each segment were constructed using

the maximum likelihood method with bootstrap values of 1,000 replicates. The bootstrap

values above 70 were indicated in each branch. The scale bars represent the number of

nucleotide substitutions per site. Serotypes of the MRVs were assigned based on the

phylogenetic tree of the S1 segment. Serotypes 1, 2, 3 and 4 were highlighted in blue, yellow,

purple and green colors, respectively.

Figure 4. Multiple amino acid sequence alignment of viral μ NS protein. (A) A schematic representation of μ NS protein encoded by M3 segment of mammalian orthoreovirus. (B) Multiple comparisons of the 250 amino acids at the C-terminal of μ NS. Amino acid sequences of μ NS of MRV12-52 and the other related strains were aligned using Clustal omega. The unique amino acids of MRV12-52 were highlighted in red color.

Figure 5. Growth efficiency of MRV12-52 in cell lines. (A) Growth kinetics of MRV12-52 in Vero E6, BHK-21, A549, Caco-2 and HEK293T cells. (B) Immunofluorescence staining of MRV12-52 in cell lines. Cells were inoculated with MRV12-52 at an MOI=0.01 and fixed at 48 hpi. Cells were stained with anti-MRV12-52 polyclonal antibody for virus antigens (green) and Hoechst 33342 for cell nuclei (blue). Scale bars =200 μ m. (C) Impact of exogenous expression of human TMPRSS2 and ACE2 on the growth kinetics of MRV12-52 in Vero E6 cells. Vero T2, Vero E6 expressing human TMPRSS2; Vero T2-hACE2, Vero E6 co-expressing human TMPRSS2 and ACE2. Cells were inoculated with MRV12-52 at an MOI of 0.01. Virus titers in the culture supernatants at 0, 24, 48 and 72 hpi were examined by FFA.

Figure 6. Replication and pathogenicity of MRV12-52 in laboratory mice. (A) Schematic overview of the experimental methods. Four-week-old, female BALB/c mice were intranasally inoculated with 10^6 ffu of MRV12-52 or PBS as control. A graphic photo was made in BioRender.com. (B) Kaplan-Meier survival analysis of mice infected with MRV12-52. A humane endpoint was considered when the mice showed more than 20% body weight loss. (C) Body weight change during the course of infection. (D) Virus titers in the lung, liver, spleen, kidney and intestine of mice at the indicated time points. (E) Viral RNA levels in organs and blood of MRV12-52 inoculated mice. Data were normalized to the mRNA levels of mouse β -

663 actin (ACTB) and the relative expression of the L2 gene was calculated by the $2^{-\Delta C_t}$ method.
664 One-way ANOVA with Dunnett's multiple comparisons was used to determine the statistical
665 significance of differences in viral titers and viral RNA levels between 1 dpi, 3 dpi, 5 dpi and
666 7 dpi. *, $p < 0.01$; **, $p < 0.001$, ***, $p < 0.0002$; ****, $p < 0.0001$.

667

668 **Figure 7. Pathological changes in the lungs of MRV12-52 infected mice.** Lungs were
669 collected from mice at 3, 5 and 7 dpi of MRV12-52 for the pathological examination. (A) Gross
670 appearance of the lungs of mice inoculated with MRV12-52 or control PBS. (B)
671 Histopathological images of the lung section by H&E staining. (C) Detection of MRV12-52
672 antigen in the lungs by IHC. Lung sections were stained with anti-MRV12-52 polyclonal
673 antibody and counterstained for cell nuclei with Hematoxylin. (D) H&E staining and IHC on
674 serial sections of the lung at 7 dpi. For IHC, the sections were stained for MRV12-52, CD3 for
675 T lymphocytes, Iba1 for macrophages, and MPO for neutrophils Scale bars in each image was
676 indicated as 1 mm or 100 μ m.

677 **Table 1.** Results of virus isolation from specimens of Indonesian fruit bats

Location	Region	Year	Bat species	Virus isolation (positive/total)		
				Lung	Throat swab	Faeces
Panjalu	West Java	2010	<i>Pteropus vampyrus</i>	0/15	-	-
Lima Puluh Kota	West Sumatra	2011	<i>Pteropus vampyrus</i>	0/20	-	-
Popayato	North Sulawesi	2011	^a <i>Pteropus</i> sp.	0/4	-	-
Paguyaman	North Sulawesi	2011	<i>Pteropus</i> sp.	0/23	-	-
		2012	<i>Pteropus</i> sp.	0/2	-	0/2
			<i>Dobsonia moluccensis</i>	0/17	-	0/17
			<i>Acerodon celebensis</i>	0/18	-	0/18
		2013	<i>Pteropus</i> sp.	0/8	0/8	0/6
			<i>Acerodon celebensis</i>	0/15	0/15	0/4
Surabaya	East Java	2012	<i>Pteropus vampyrus</i>	0/3	-	0/3
Magelang	Central Java	2012	<i>Pteropus vampyrus</i>	0/20	-	3/19
Soppeng	South Sulawesi	2014	<i>Pteropus</i> sp.	0/7	0/7	0/7
Sidrap	South Sulawesi	2014	<i>Pteropus</i> sp.	0/15	0/15	0/15
Total				0/167	0/45	3/91

678 ^a*Pteropus* sp.; bat genetically closely related to *P. hypomelanus*

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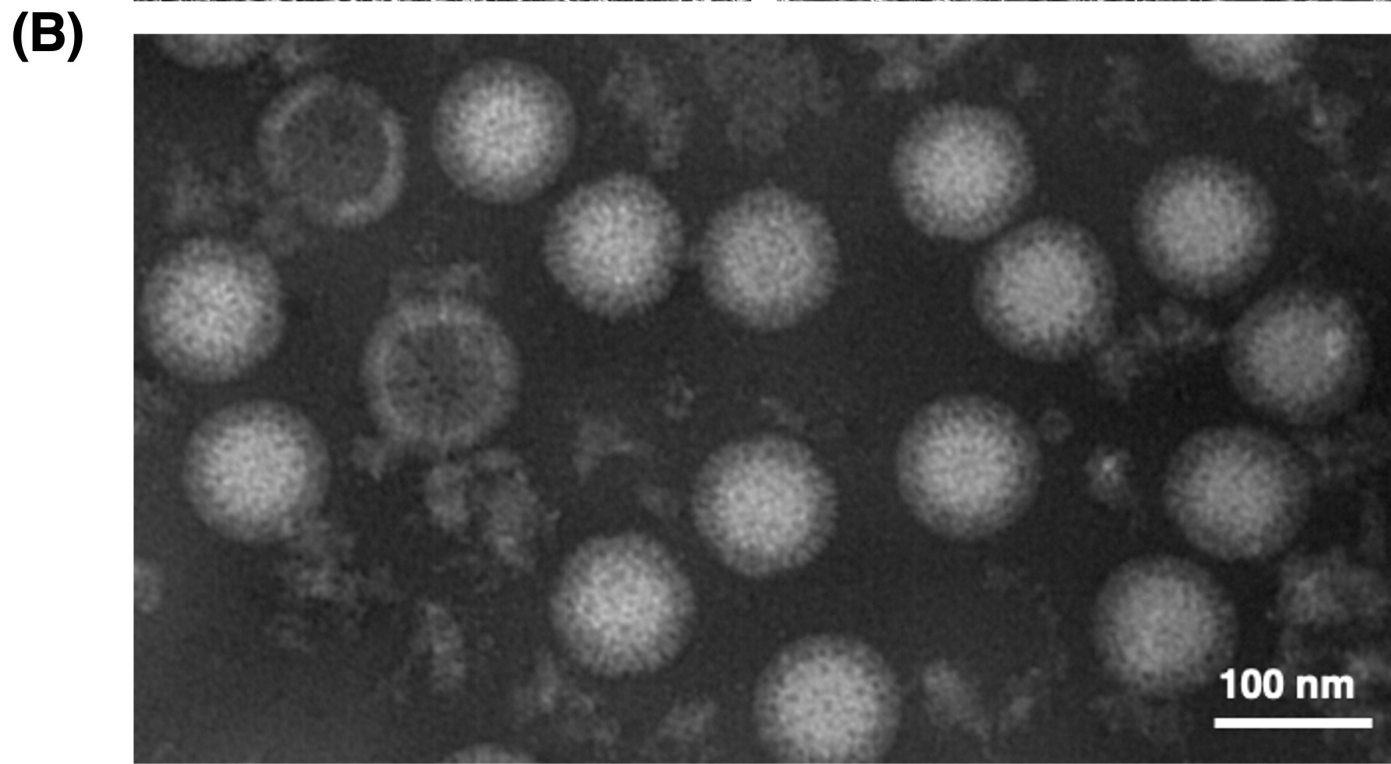
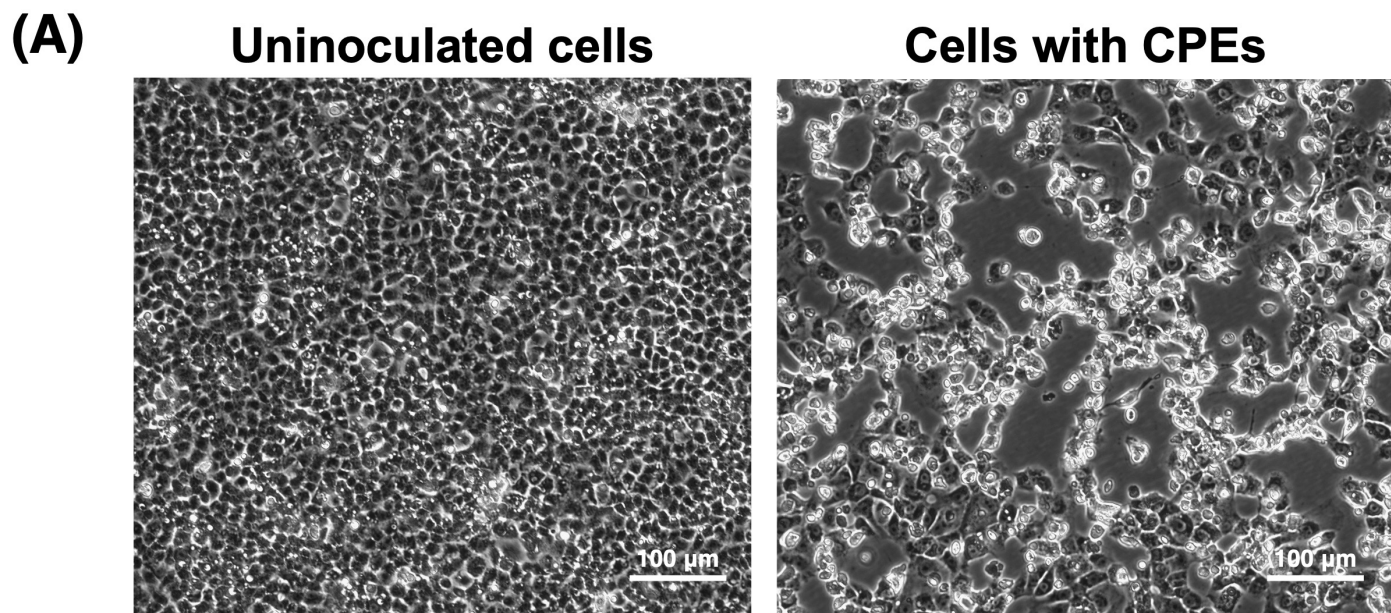
Table 2.

Pairwise comparison of coding nucleotide sequence and amino acid sequence of 10 genome segments of MRV12-52 with the prototype, human, pig, bat, and wildlife MRVs

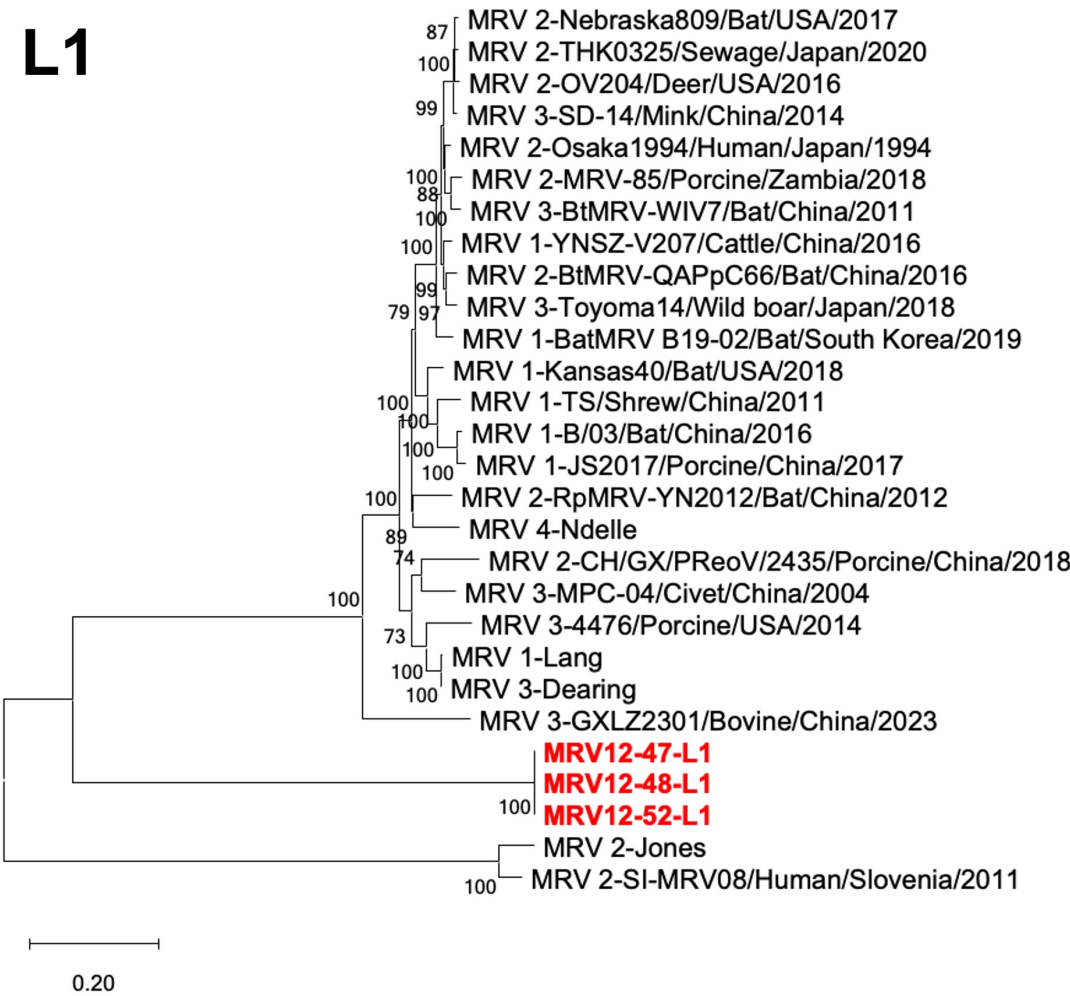
MRV12-52	Nucleotide and amino acid sequence identity											
	Prototype strains				Human		Pig		Bat		Wildlife	
	Lang	Jones	Dearing	Ndelle	SI-MRV08	Osaka 1994	4476	CH/Gx/PRco	Kansas40	YN2012	SD-14	OV204
L1 (λ3)	75.8 (91.9)	74.5 (91.0)	75.8 (92.4)	75.6 (91.9)	73.9 (91.1)	76.5 (92.7)	76.0 (92.3)	76.7 (92.9)	76.6 (92.5)	76.0 (92.7)	76.4 (92.8)	76.4 (92.7)
L2 (λ2)	73.3 (88.1)	73.0 (87.3)	75.2 (89.5)	^a NA	73.0 (87.0)	73.5 (88.1)	73.8 (87.4)	73.6 (87.4)	75.3 (89.1)	74.6 (89.4)	73.4 (88.3)	73.1 (88.3)
L3 (λ1)	77.1 (96.1)	77.4 (95.5)	76.8 (96.1)	NA	77.5 (95.3)	77.3 (96.6)	76.7 (95.9)	77.1 (96.0)	77.3 (96.6)	77.2 (96.0)	77.4 (96.7)	77.2 (96.8)
M1 (μ2)	72.9 (82.9)	71.4 (80.6)	72.6 (82.5)	NA	69.9 (80.0)	72.4 (82.5)	72.1 (82.3)	71.6 (81.7)	72.6 (82.7)	71.9 (81.9)	71.9 (82.3)	72.1 (83.6)
M2 (μ1)	79.0 (97.6)	78.2 (97.2)	79.0 (97.0)	79.3 (98.2)	77.8 (96.9)	80.3 (97.5)	79.0 (97.0)	78.9 (96.6)	79.3 (97.7)	80.2 (97.0)	80.7 (97.7)	80.3 (97.6)
M3 (μNS)	72.7 (83.8)	70.5 (80.3)	72.2 (83.8)	NA	71.6 (80.8)	72.8 (83.9)	71.6 (83.1)	72.6 (82.9)	72.9 (83.1)	72.8 (83.9)	72.2 (83.9)	71.7 (83.1)
S1 (σ1)	57.1 (51.8)	61.7 (62.8)	38.0 (25.7)	37.2 (24.2)	60.1 (60.8)	70.8 (74.6)	36.9 (24.5)	65.3 (68.8)	56.9 (52.2)	91.6 (95.2)	38.0 (21.3)	93.6 (97.0)
S2 (σ2)	77.7 (95.9)	77.2 (94.3)	78.5 (95.5)	76.7 (94.0)	77.5 (93.8)	76.8 (95.7)	76.7 (95.2)	77.1 (95.5)	77.7 (95.9)	77.5 (95.7)	77.0 (95.9)	76.1 (95.9)
S3 (σNS)	77.5 (89.6)	74.8 (86.1)	76.1 (89.9)	NA	74.8 (87.2)	77.2 (90.4)	77.8 (91.0)	76.5 (90.4)	76.7 (90.2)	77.8 (91.0)	77.2 (90.2)	76.3 (89.6)
S4 (σ3)	87.0 (96.2)	80.0 (91.2)	87.5 (96.4)	89.7 (95.8)	80.4 (90.7)	95.1 (98.4)	76.2 (86.0)	77.5 (85.8)	92.5 (97.0)	91.1 (97.3)	93.6 (97.8)	93.7 (97.8)

682

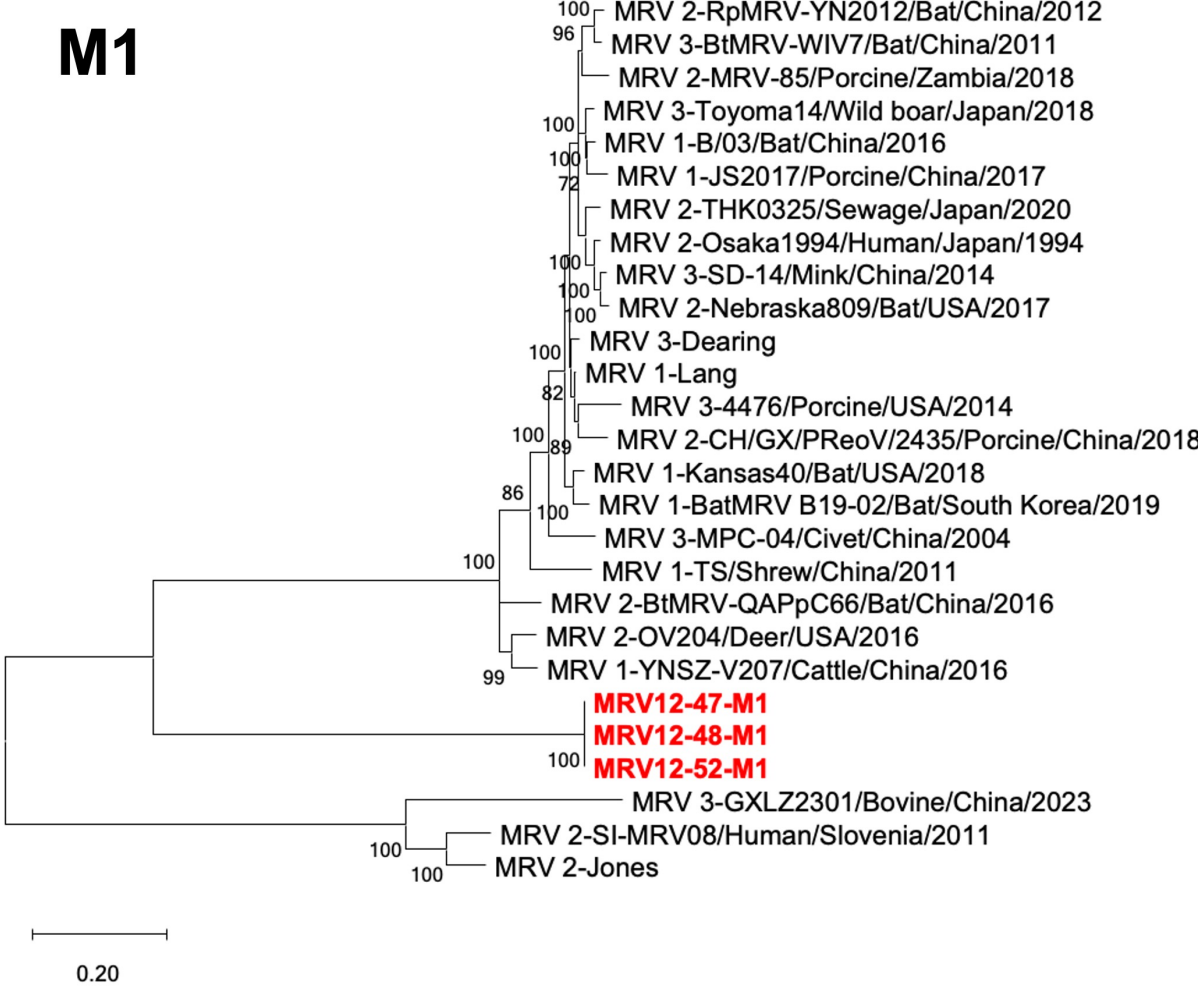
^aNA; sequence is not available, Number in bold refer to the highest nucleotide sequence identity to those of MRV12-52 and its amino acid sequence identity in parenthesis



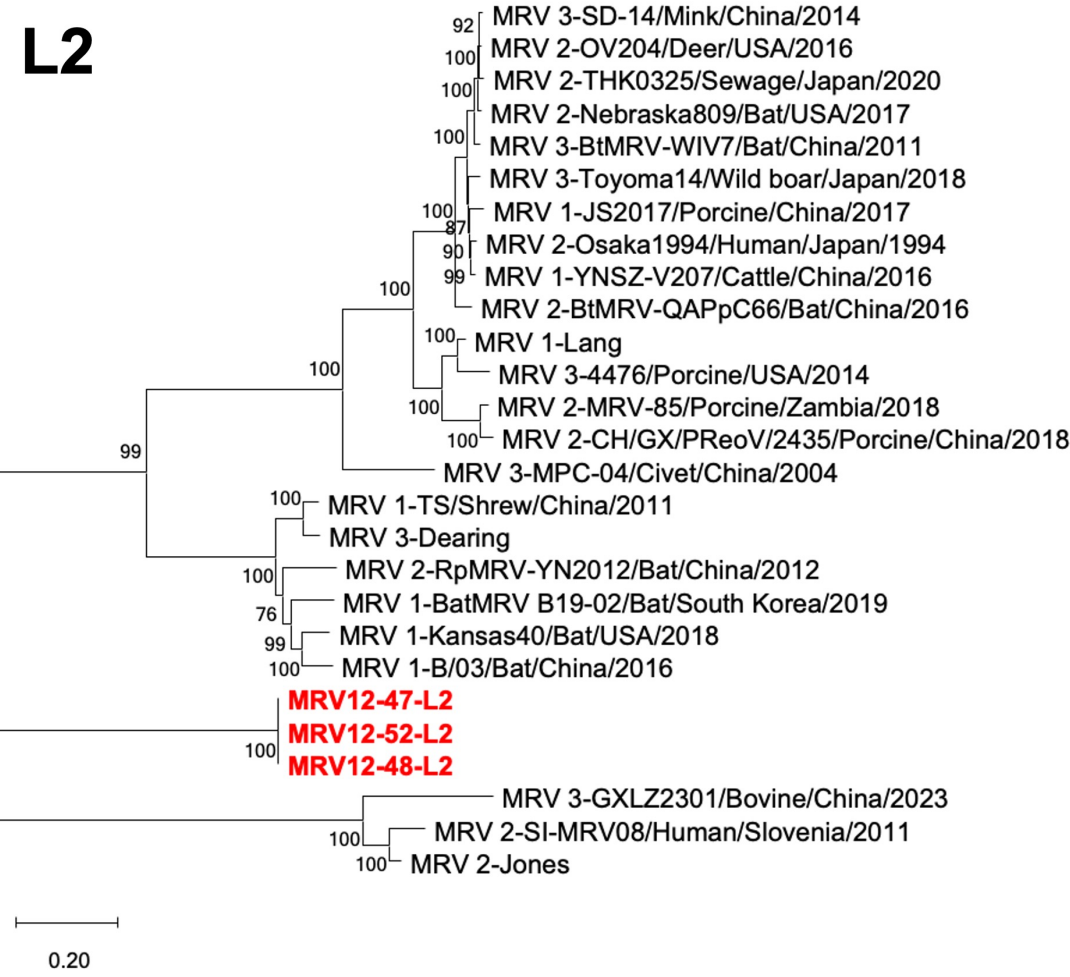
L1



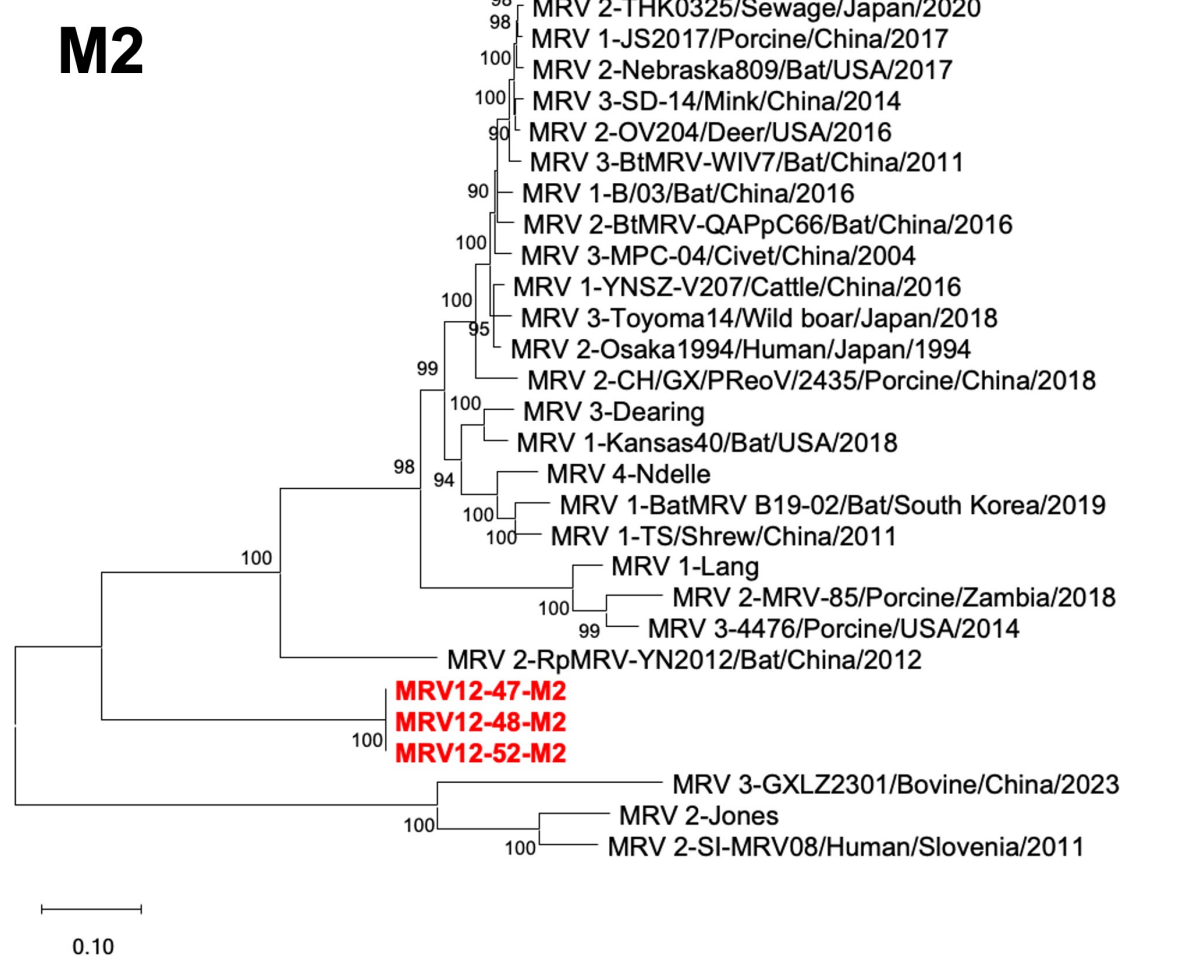
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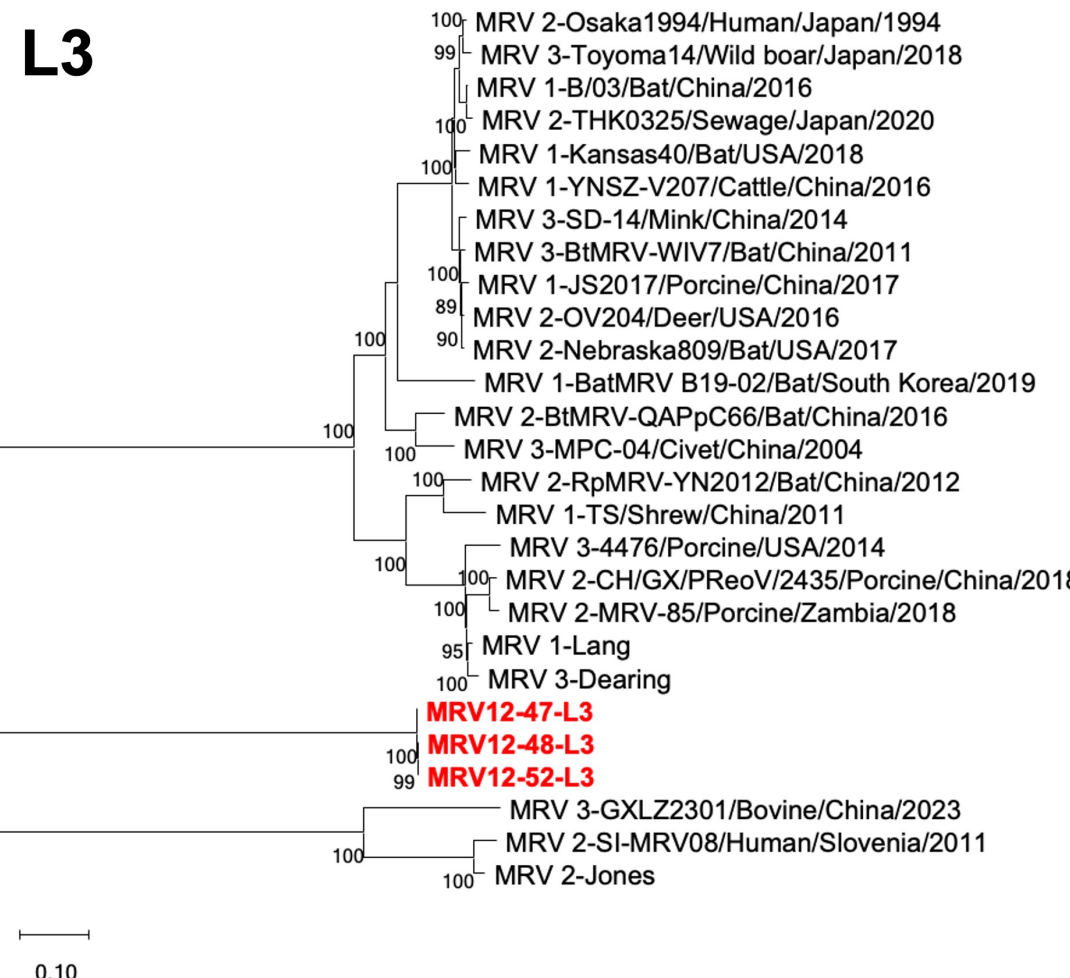
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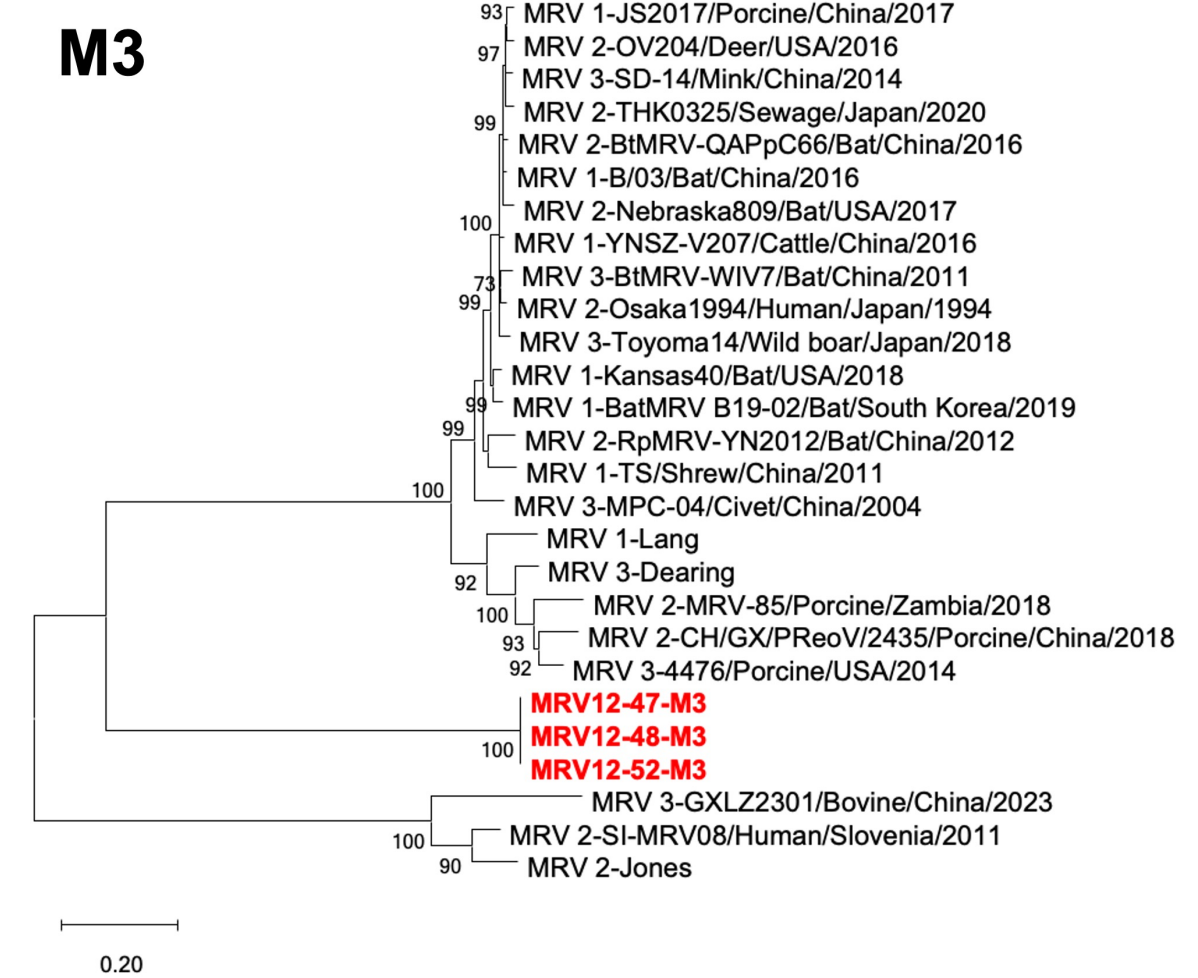
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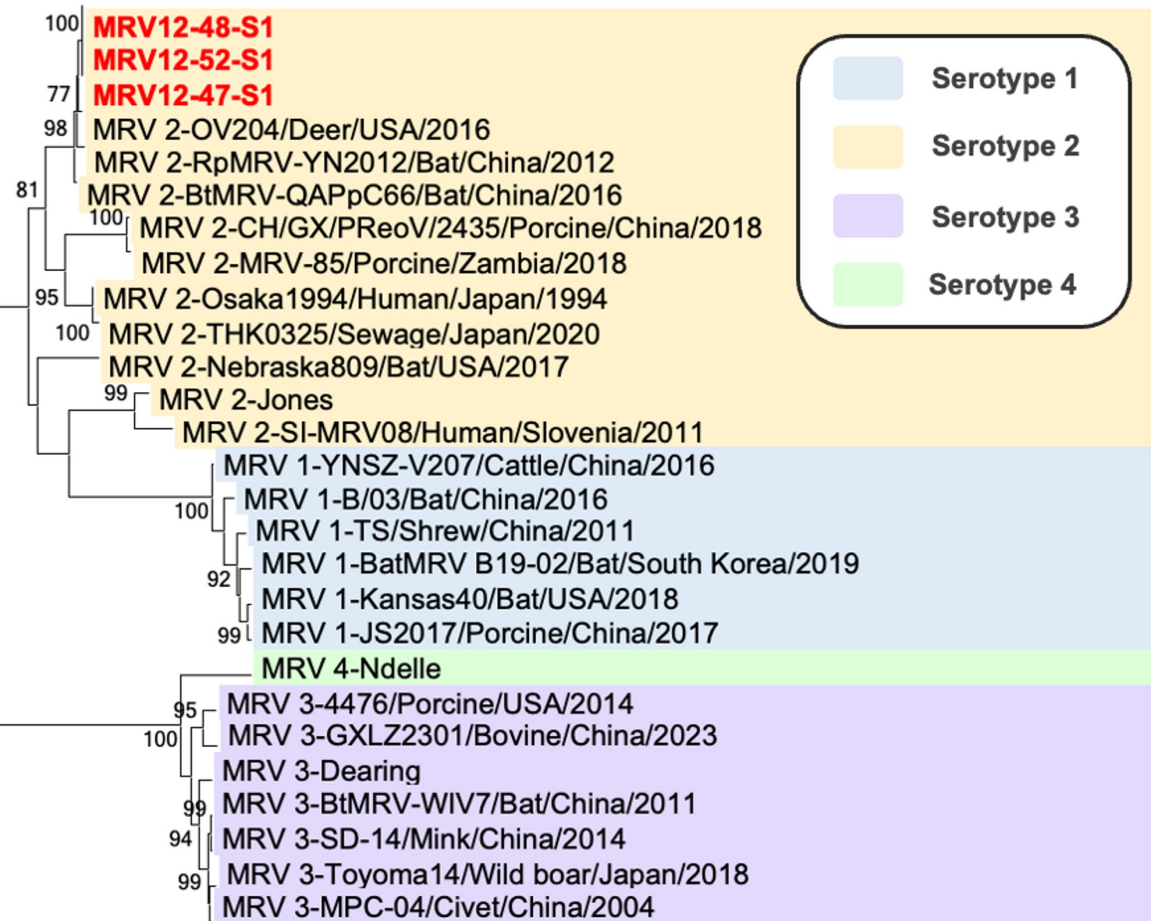
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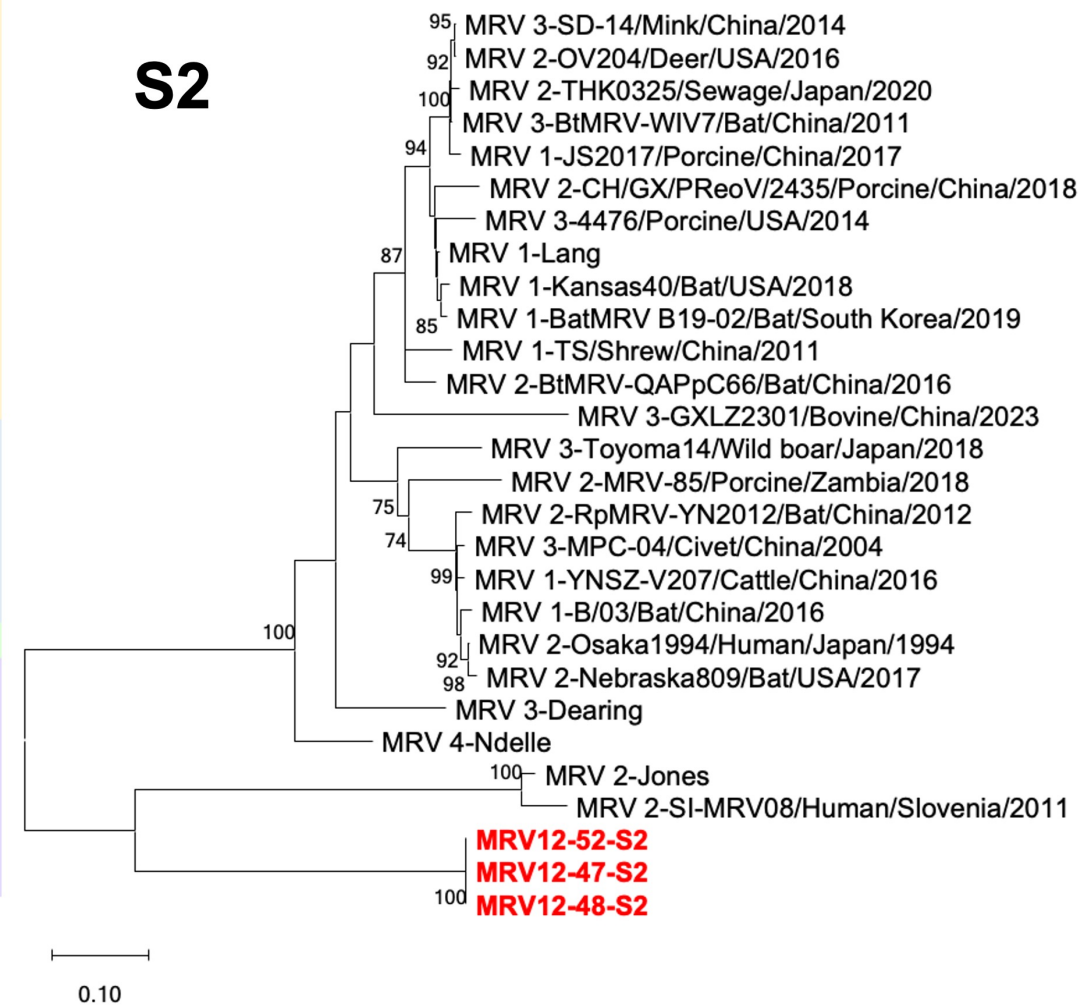
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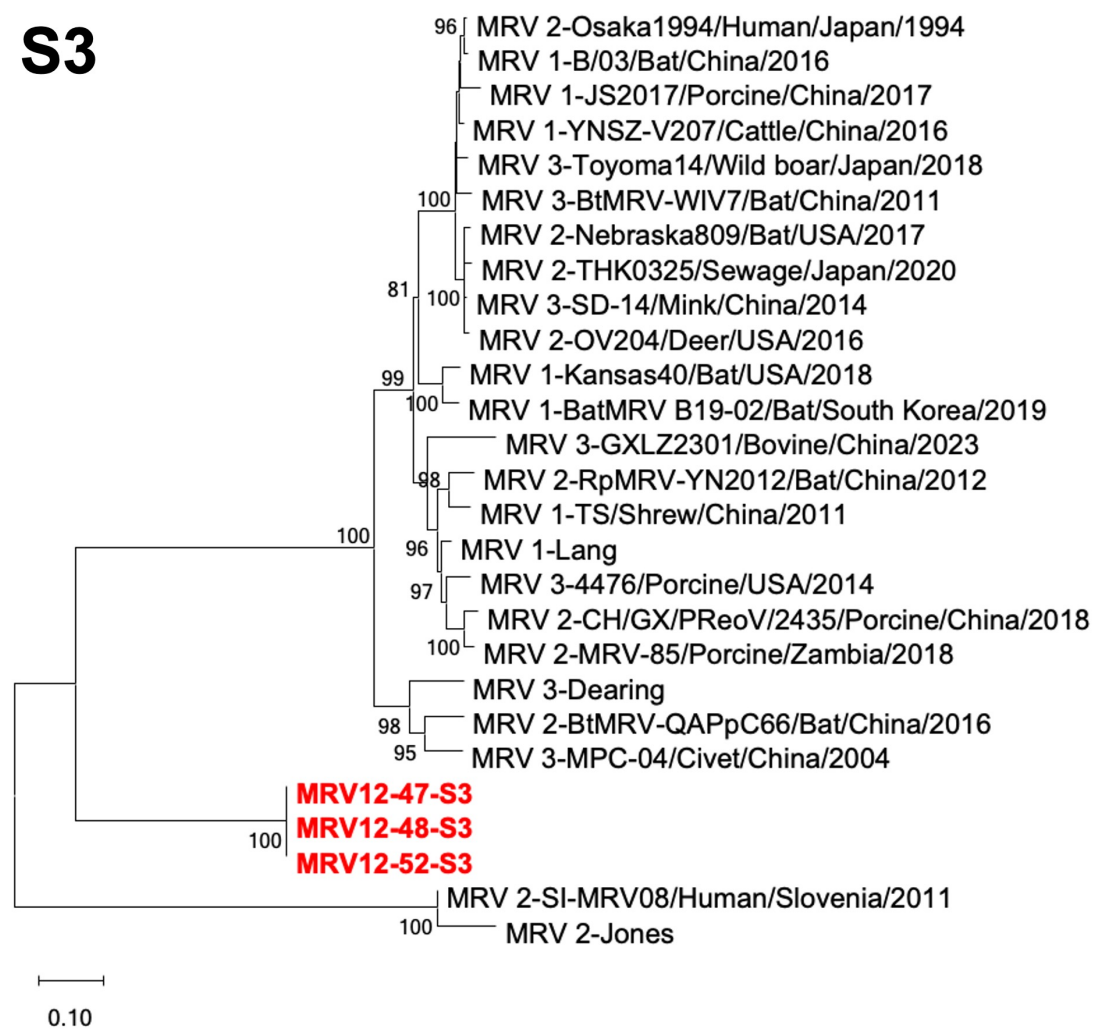
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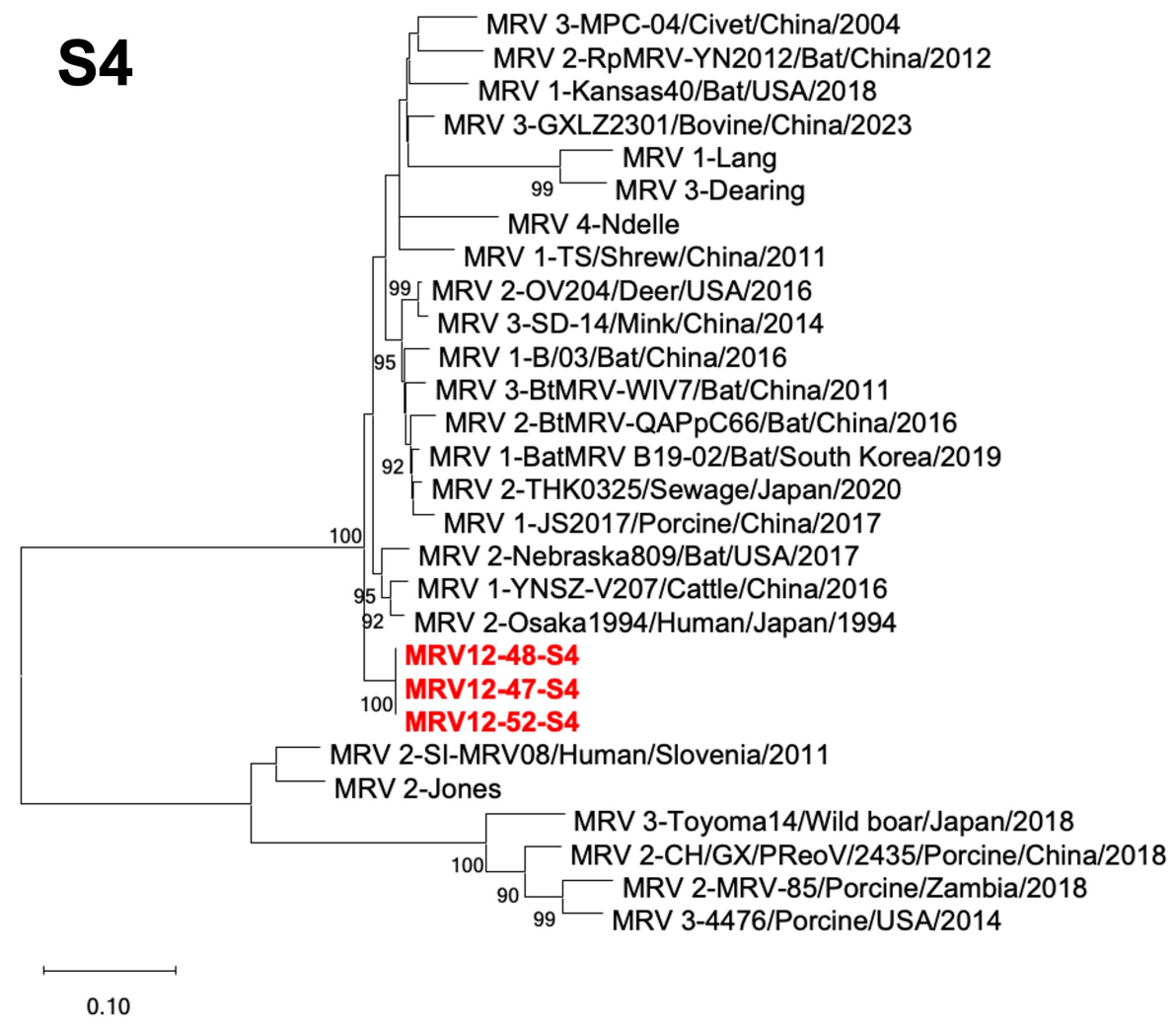
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S3

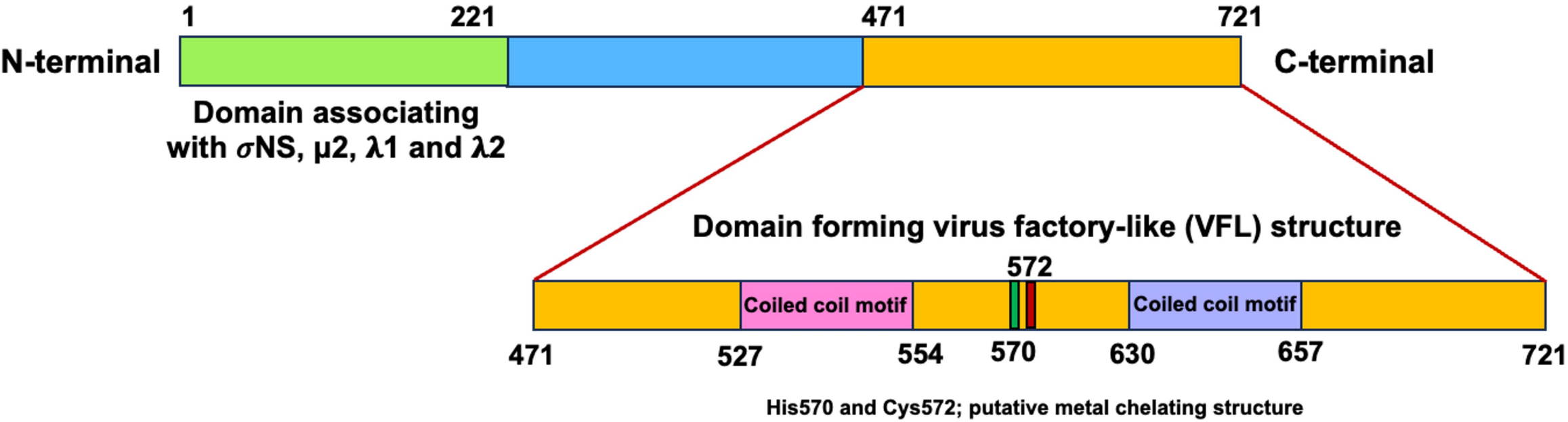


S4



(A)

Structure of μNS protein

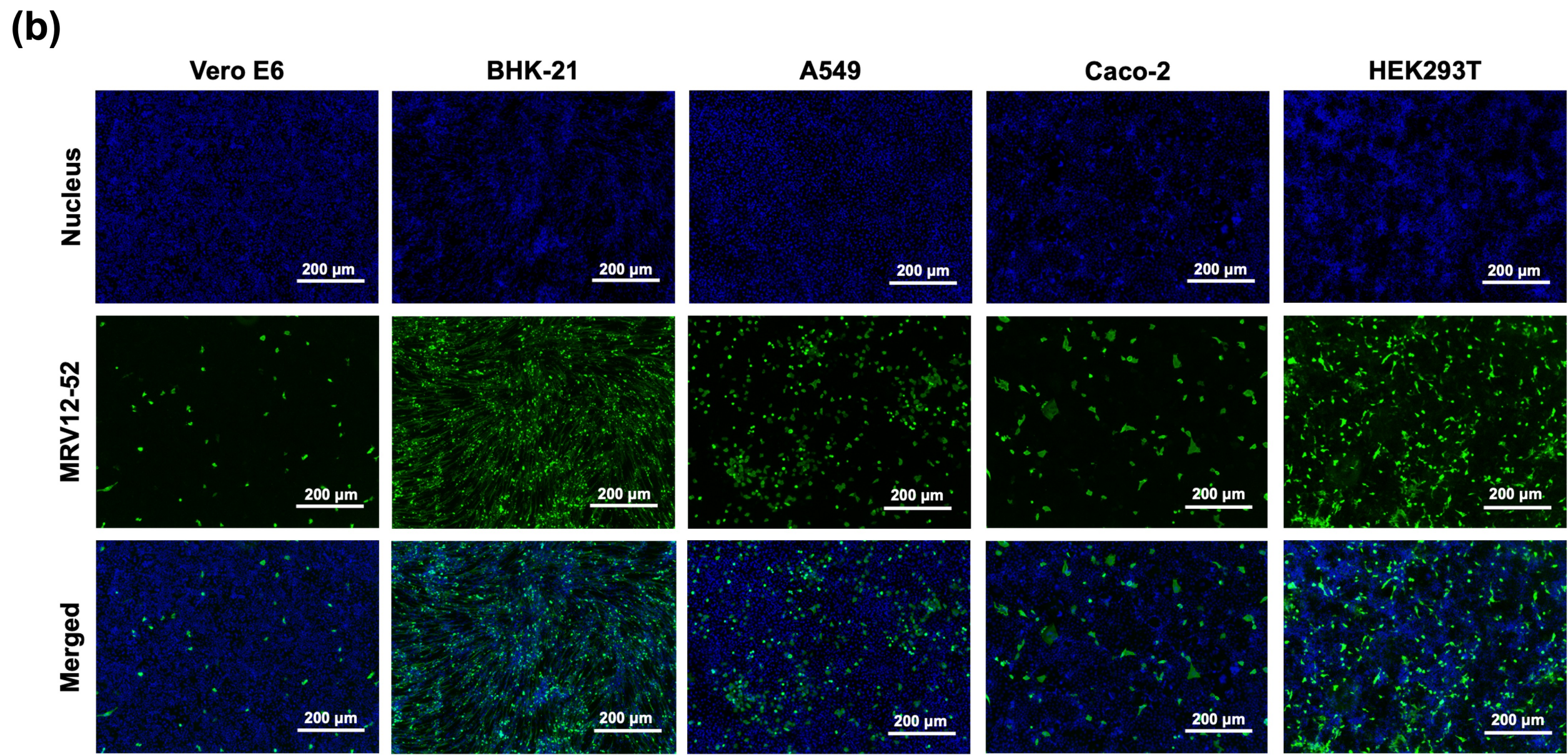
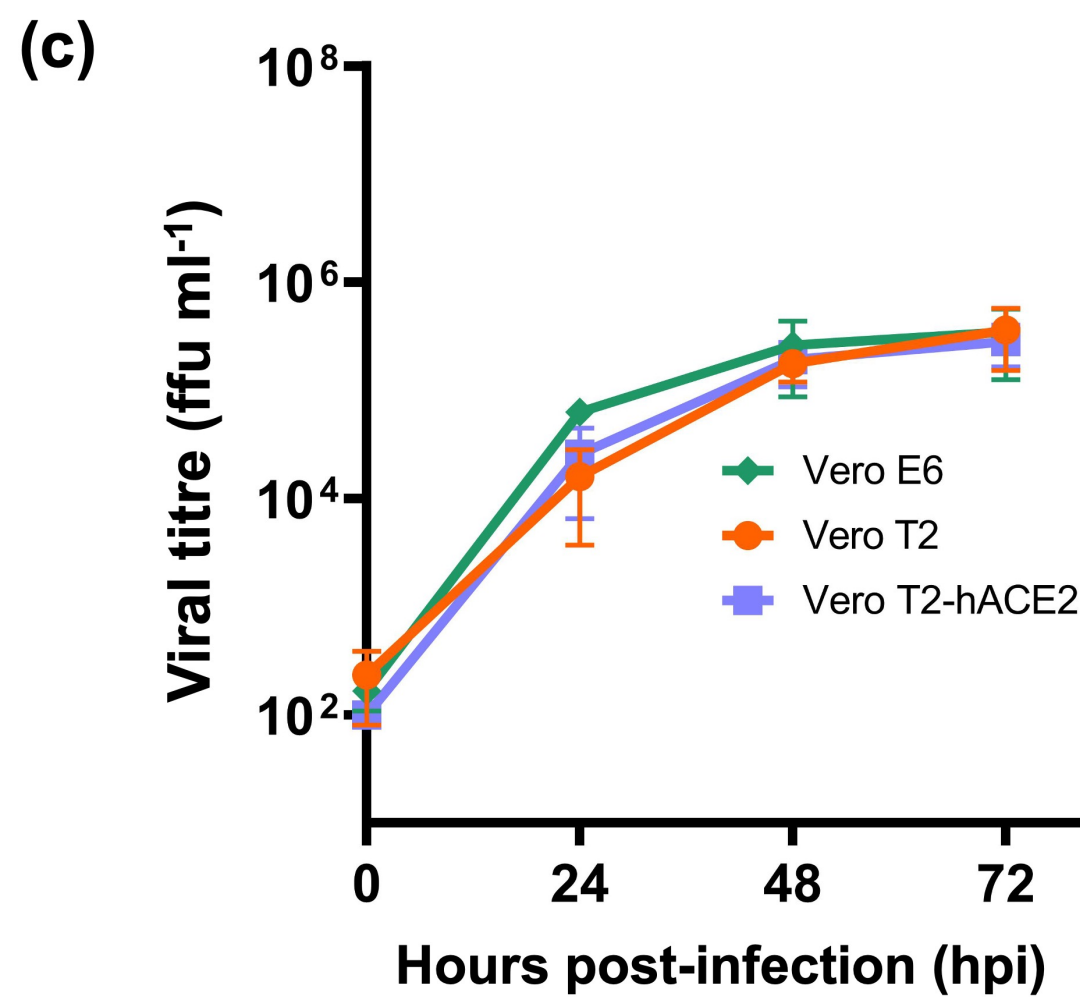
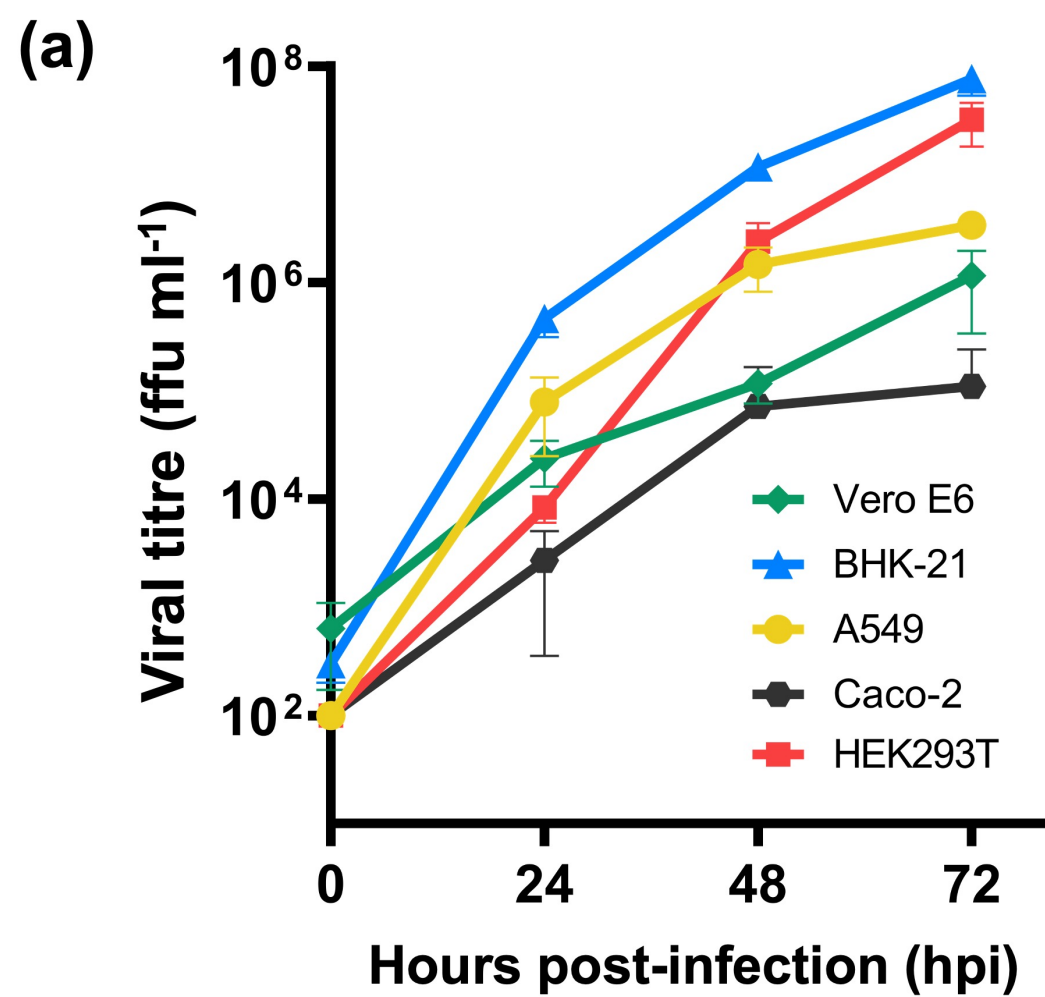


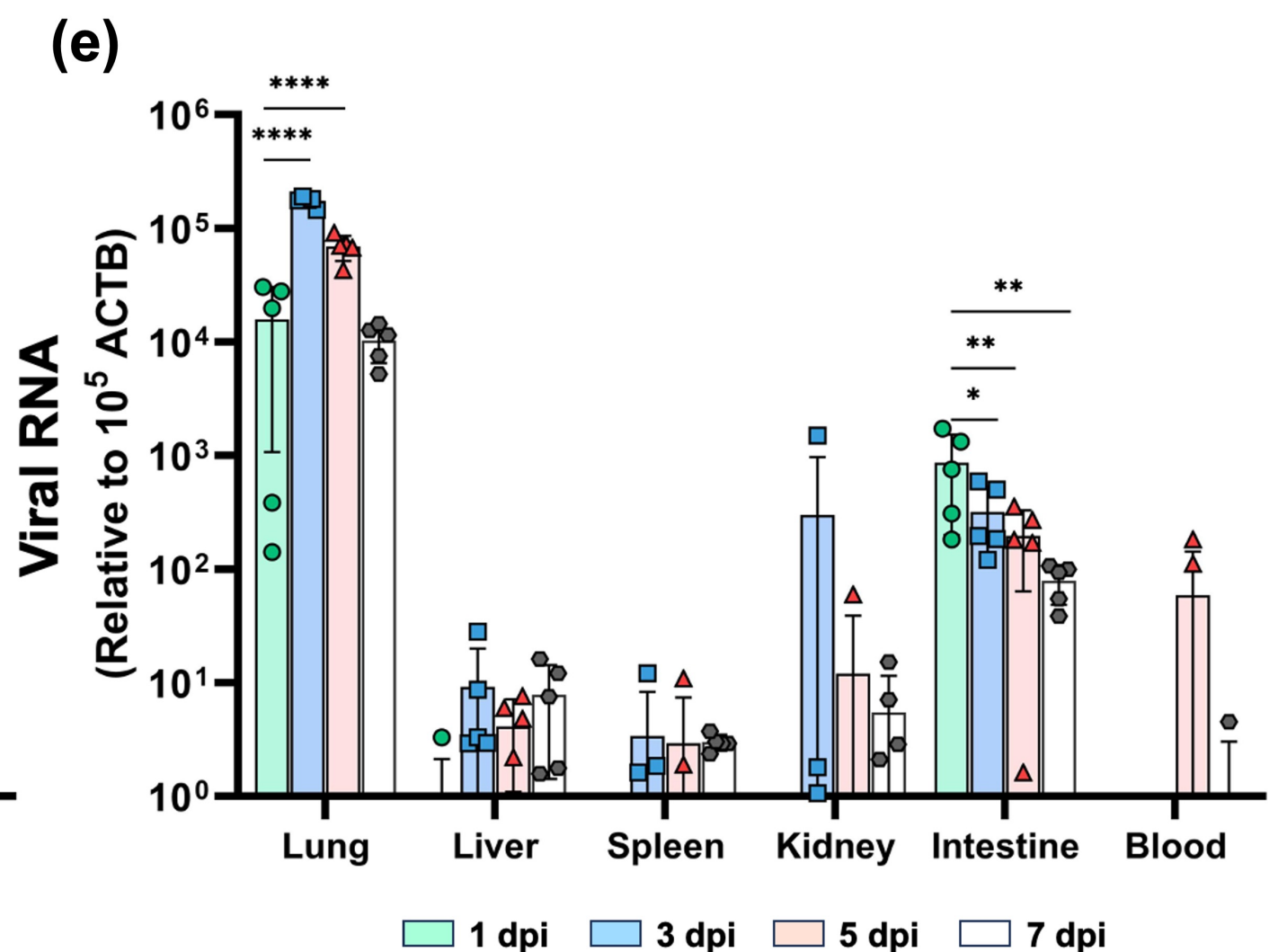
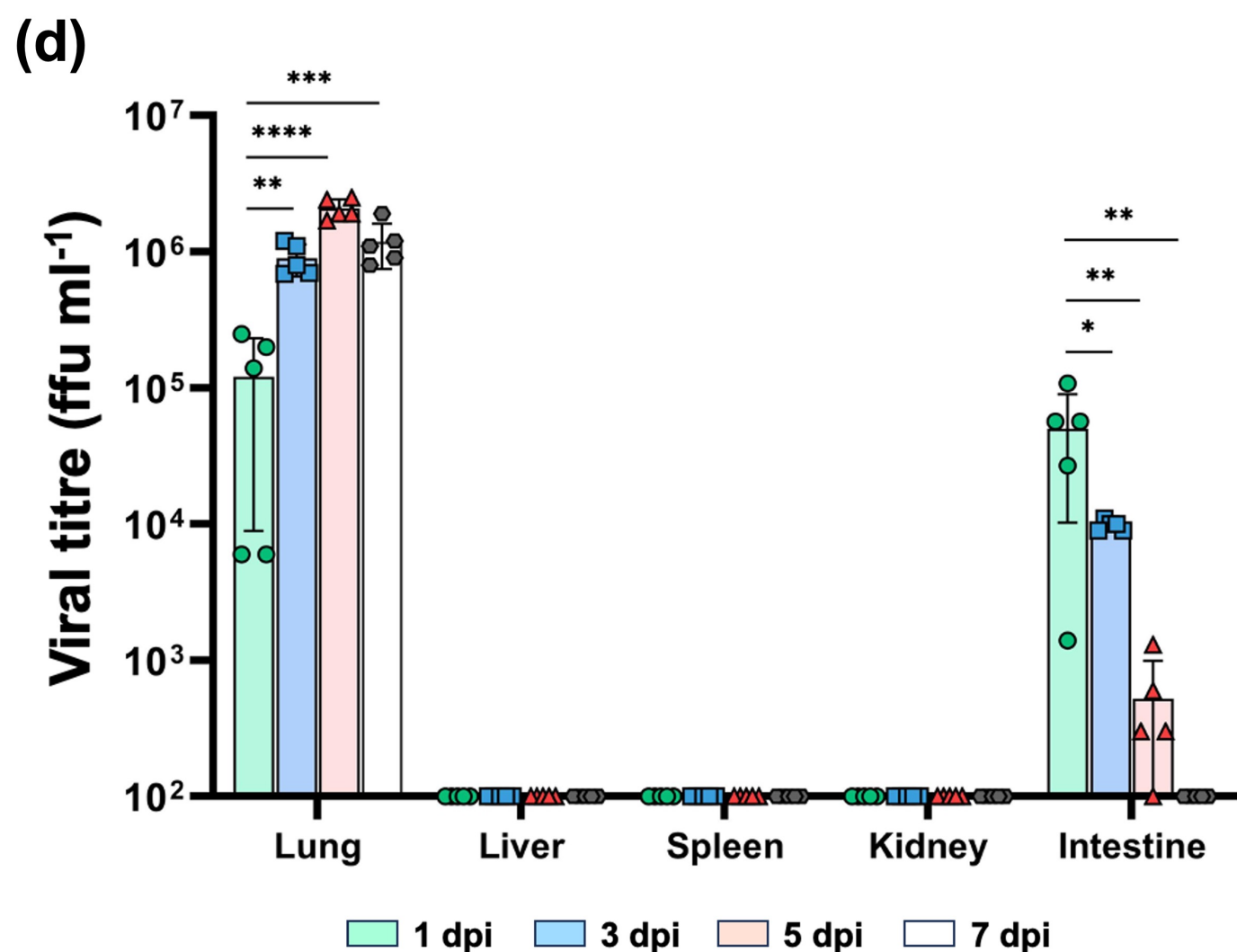
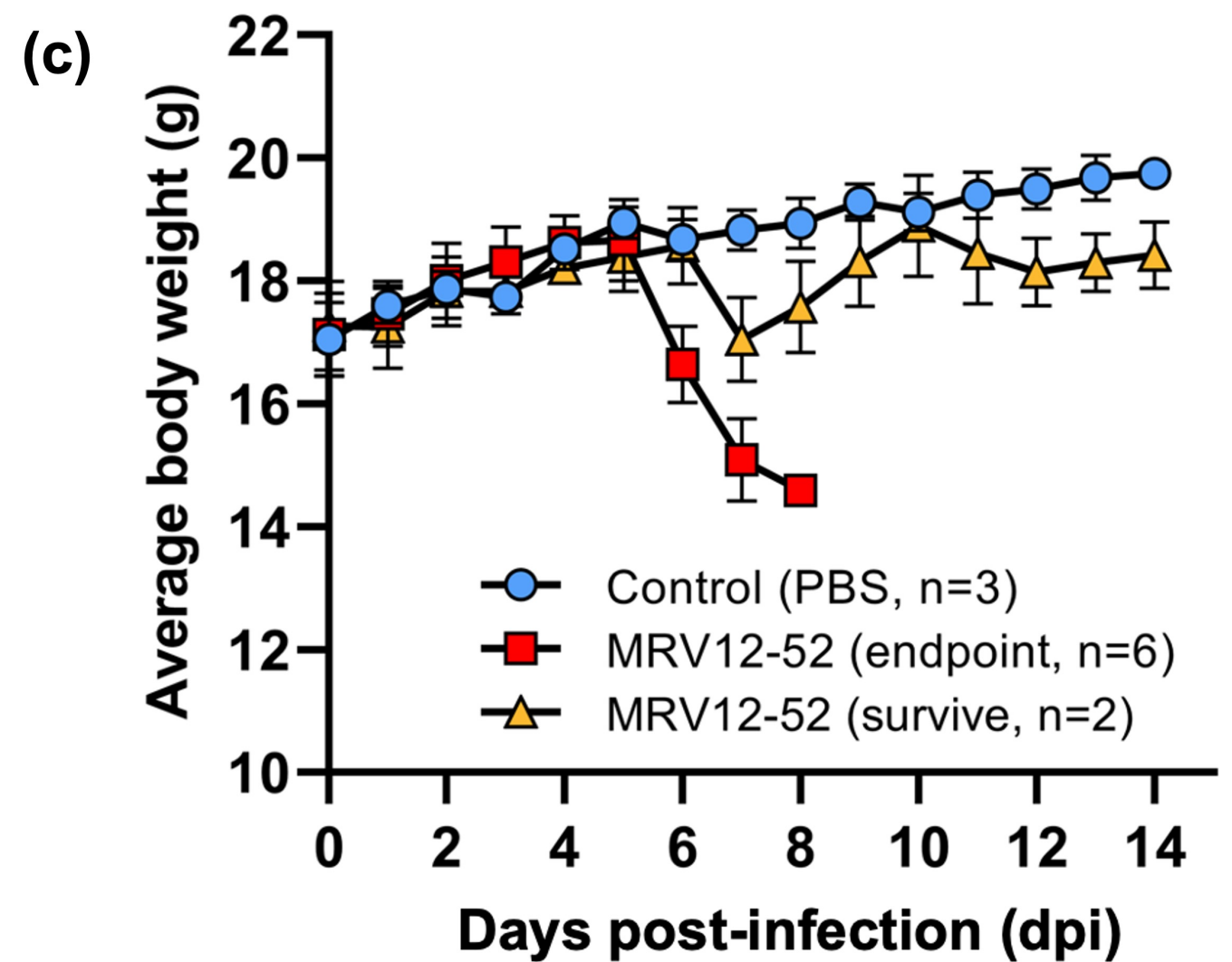
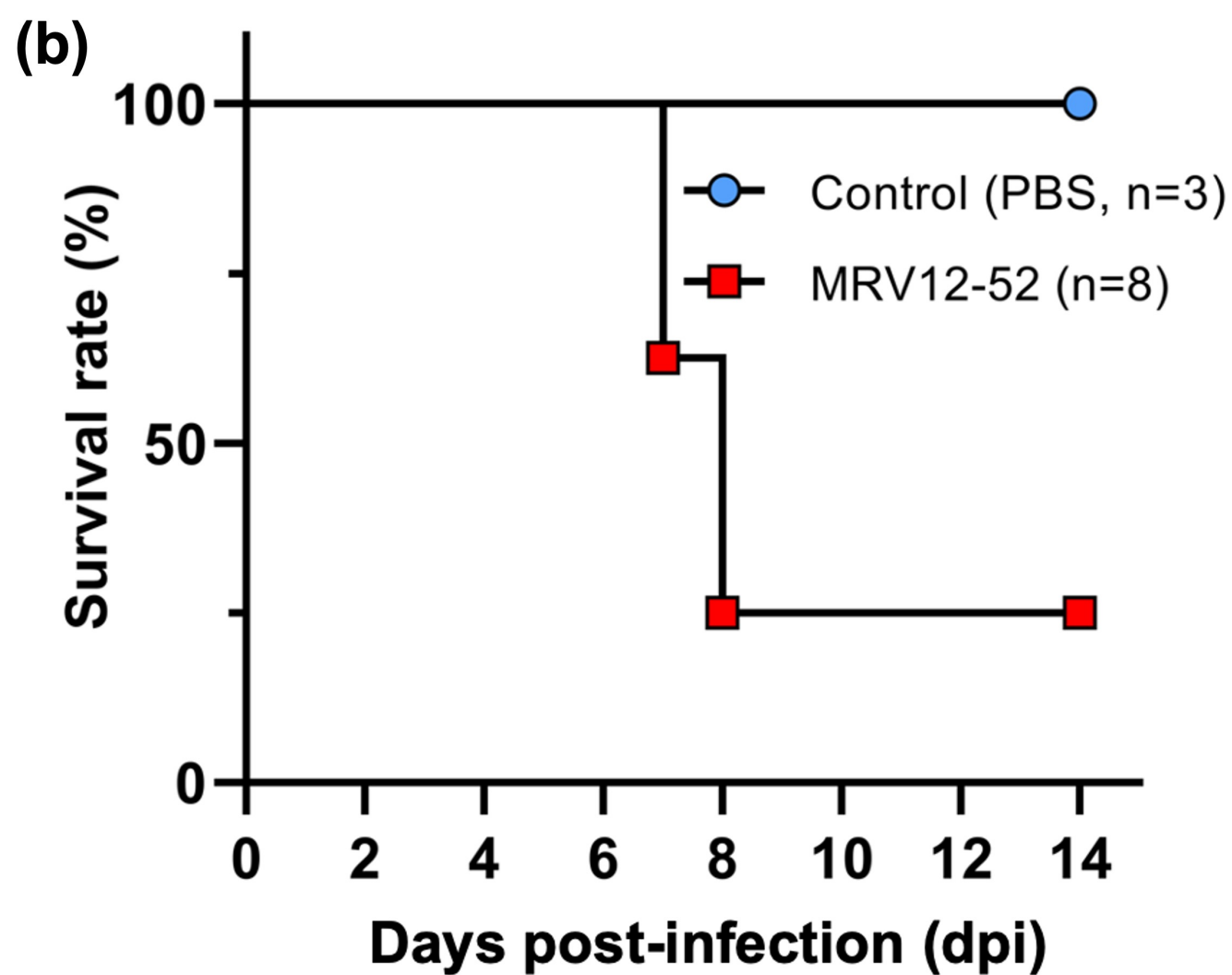
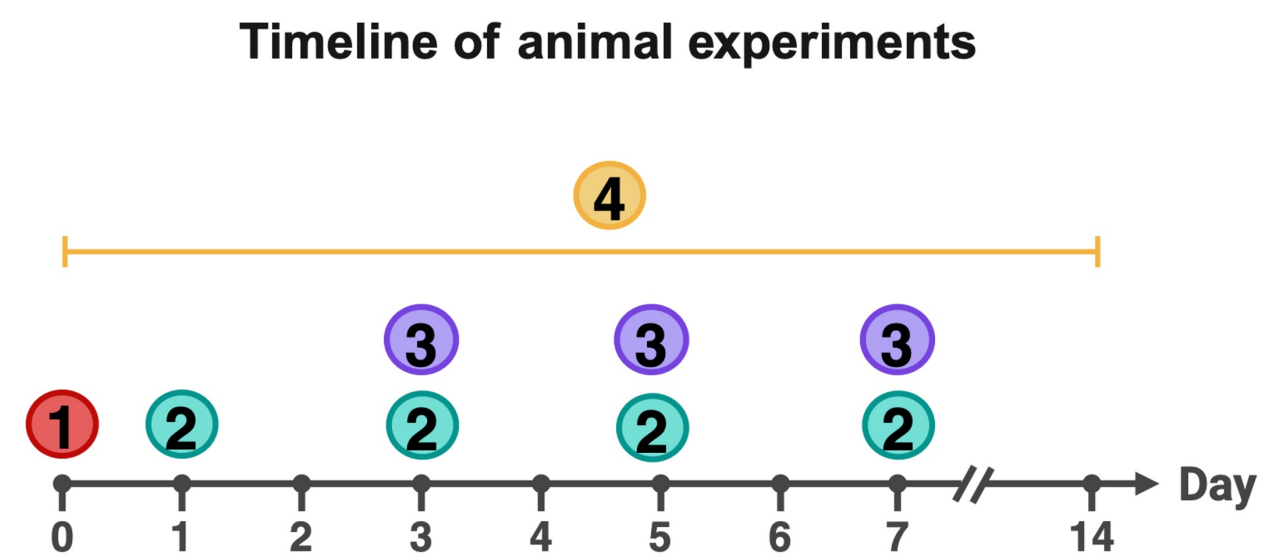
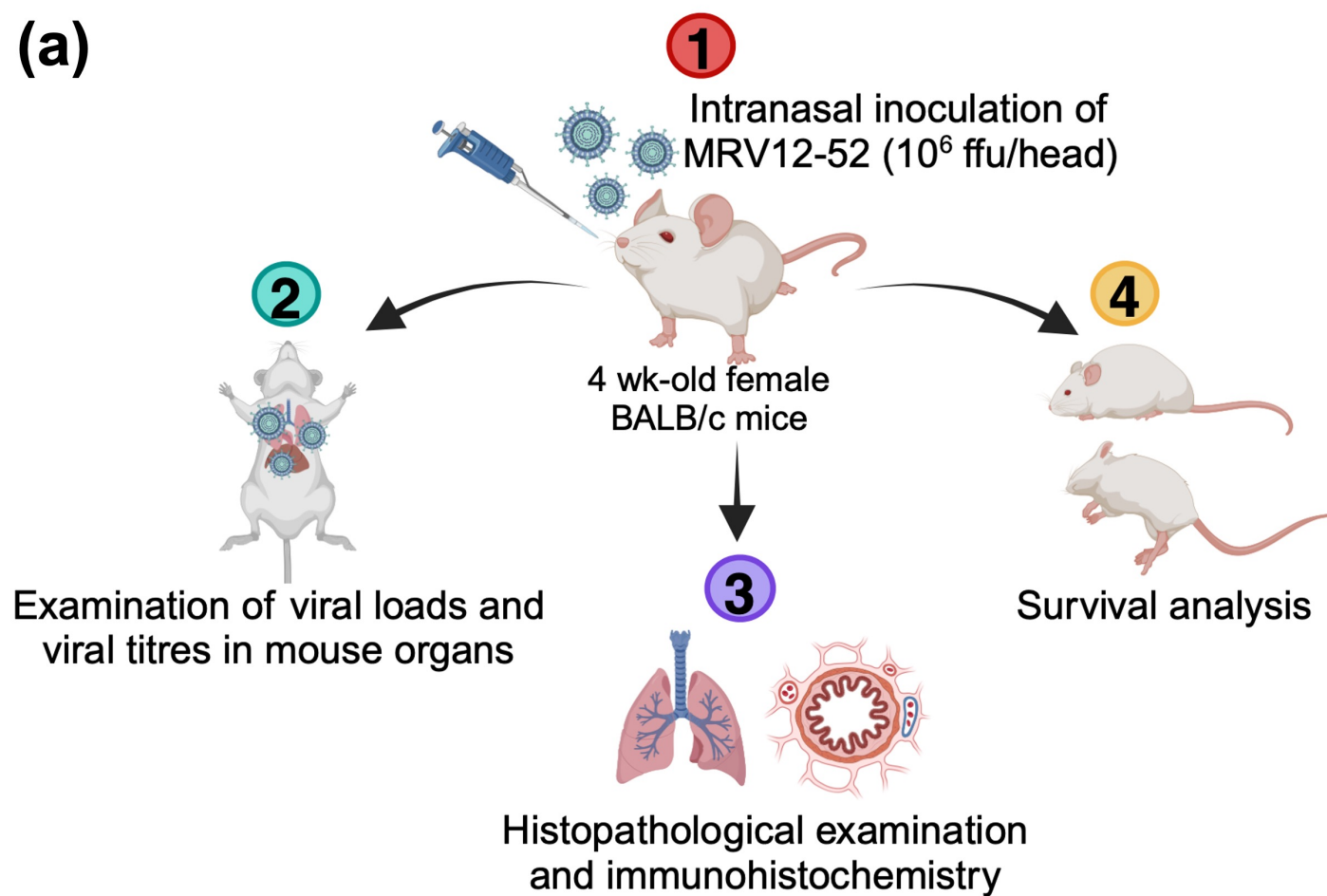
(B)

		470	480	490	500	510	520	530	540	550	
Consensus	LFLERXNDMVDGIKLQLDASRQC	HECPVLQQKVVELEKQIIMQKSIQSDPTPMALQPLLSQLRELSSEVTRLQMELSRAQSLNAQLEADVKS	A								558
Bat/MRV12-52	.YS.K...T.....	I			L.V.R.F.T.T.....					I.....M...	558
Type 1 Lang	S.....									T.....A...	558
Type 2 Jones	..MSS.S.T.....	L...I...HL.V...AS...V.....							D...T.AI.TR.....		558
Type 3 Dearing	VT.....						V.....				558
Human/SI-MRV08	..MSS.S.T.....	L...I...HM.V...TS...V.....							T.AI.TR.....		557
Pig/4476	.LP....V.....	H.....V.....									558
Pig/CH/Gx/PReo	.LS....V.....	H.....V.....									558
Bat/Kansas40	...D.....				V.....		T.....	G.I.....			558
Bat/YN2012								G.....			558
Mink/SD-14	...D.....										558
Deer/OV204	...D.....							G.....			558

		560	570	580	590	600	610	620	630	640	650	
Consensus	QSCSLDMYLRHHTCINGHAK	EDLLDAVRVAPDVRREIMEKRSEVRKGCERISKEXAAK	CQTVIDDLTLMNGKQAREITELRESAENYEKQI								651	
Bat/MRV12-52	.A.....	I.V.....	SL..KD.LK.ET..QE.WD..A...SD.FSNQMEE..V...R..Q..AT..AVDT...								V	651
Type 1 Lang	.A.....	T.....	K.....G...R.....Q.....									651
Type 2 Jones	K.....	S.V...M...I...Q.L.L.KAT.QE.W...AR..STTF.SK..E.....H..S...D.VT....V										651
Type 3 Dearing						Q.....				Q.....D...K....		651
Human/SI-MRV08	S.....					Q.L.L.KAT.QE.W...A...STTF.SK..E.....H..S...D.VT....V						650
Pig/4476						Q...R.....S.E.....E.....Q..I..D.....						651
Pig/CH/Gx/PReo	V.....					R.....S...M.E.....Q..I..D.....						651
Bat/Kansas40							R.....Q.....					651
Bat/YN2012					I.....		R.....A...Q.....					651
Mink/SD-14								HT.....V.....				651
Deer/OV204					I.....			Q.....				651

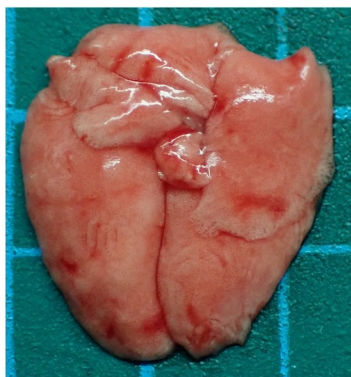
		660	670	680	690	700	710	720	
Consensus	AELVSTITQNQITYQQELQALVAKNVELDTLNQRQAKSLRITPSLLSATPIDSV	DGAADLIDFSVPTDEL							721
Bat/MRV12-52	S...N...V.....	L..A...R.M...T...PQNTT...SP...							721
Type 1 Lang	...G...M.....	M.....							721
Type 2 Jones	T.....	I..A...V..S...A...S...A...							721
Type 3 Dearing			A.....			A..V.....			721
Human/SI-MRV08	...N...K.....	I.....V..S...A...S...AA...							720
Pig/4476			R.....V..T.....						721
Pig/CH/Gx/PReo			R.....V..T.....						721
Bat/Kansas40			A.....			I..T.....			721
Bat/YN2012						T.....			721
Mink/SD-14						ST.....			721
Deer/OV204						SN.....			721



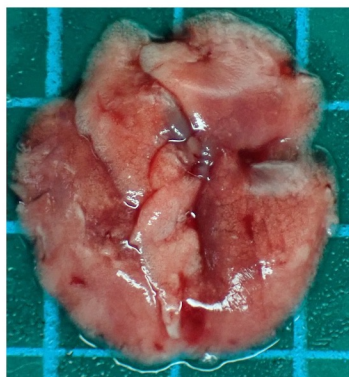


(A)

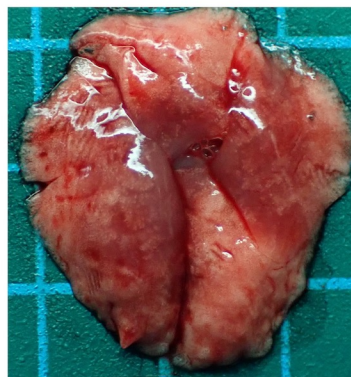
Uninfected



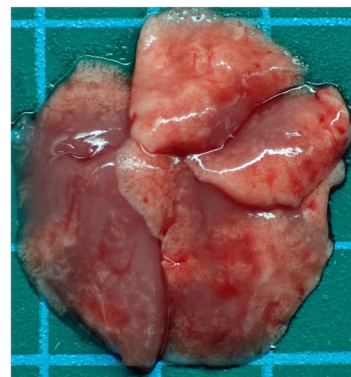
3 dpi



5 dpi



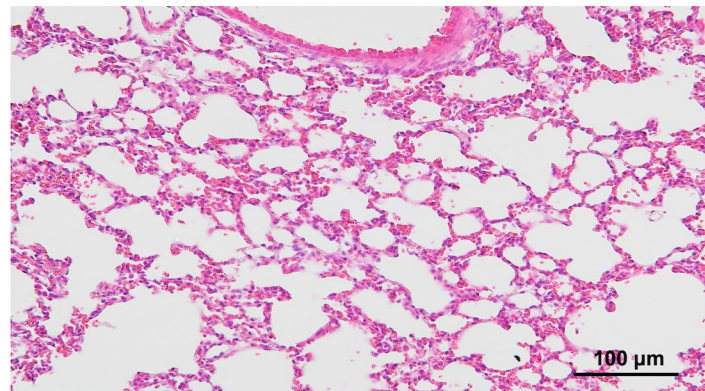
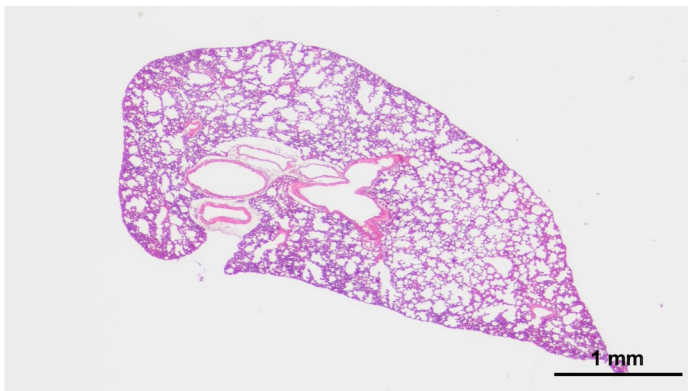
7 dpi



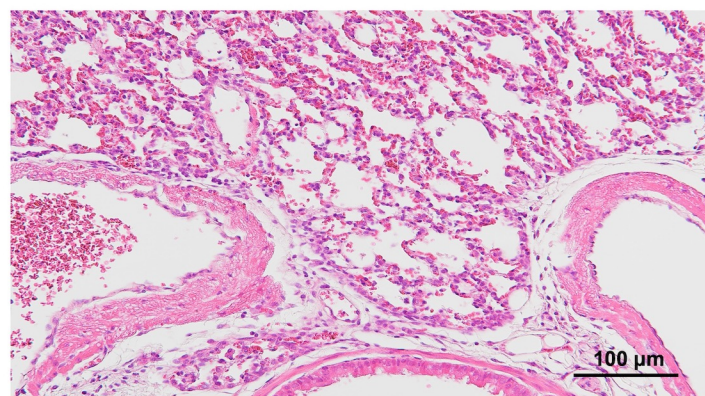
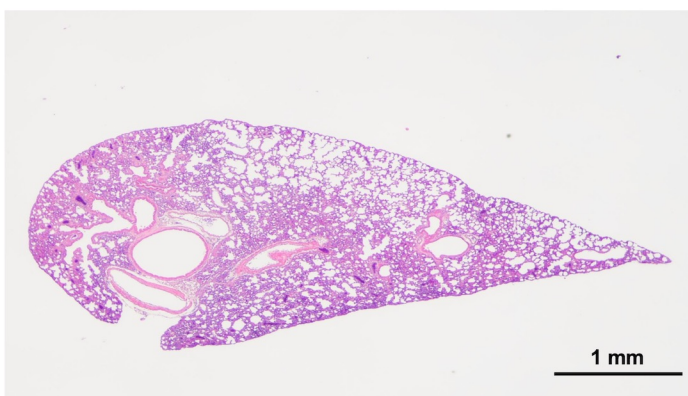
(B)

H&E

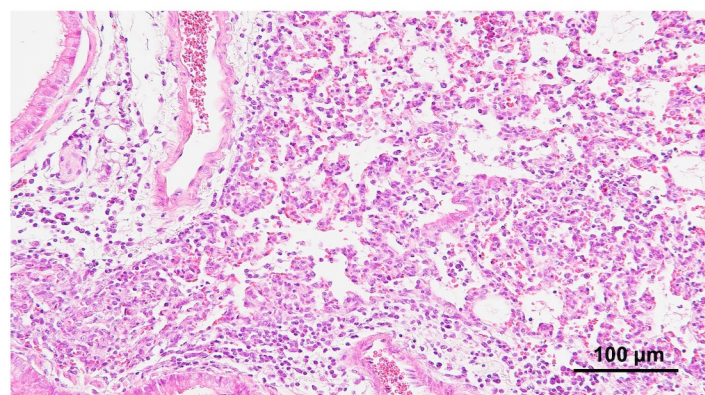
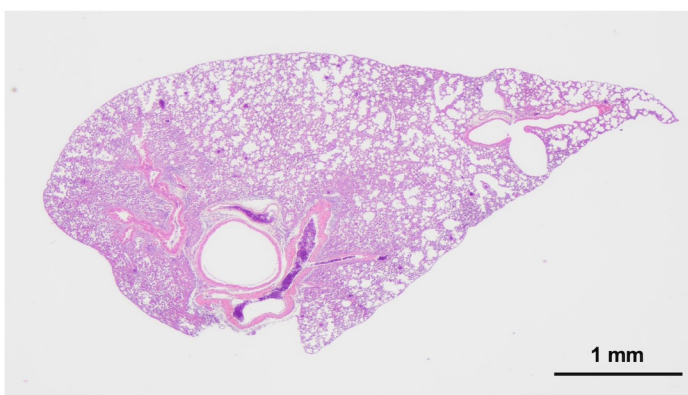
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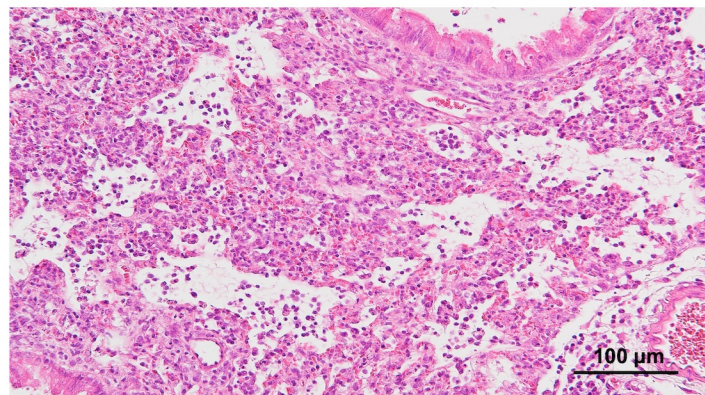
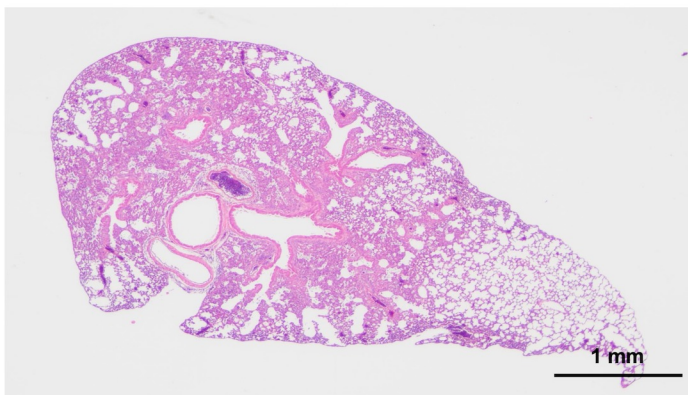
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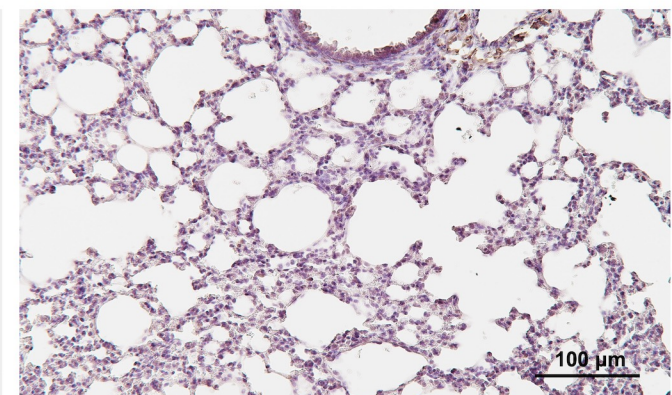
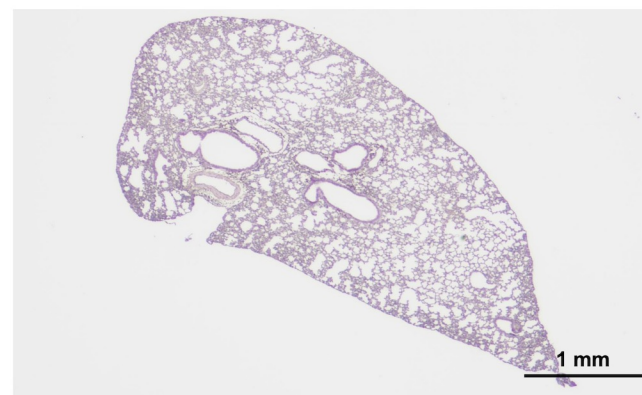
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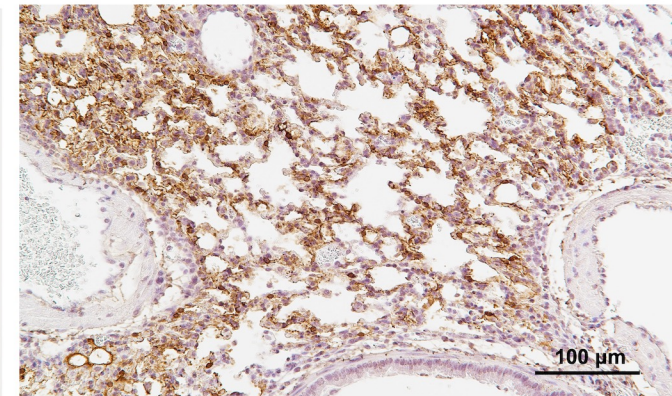
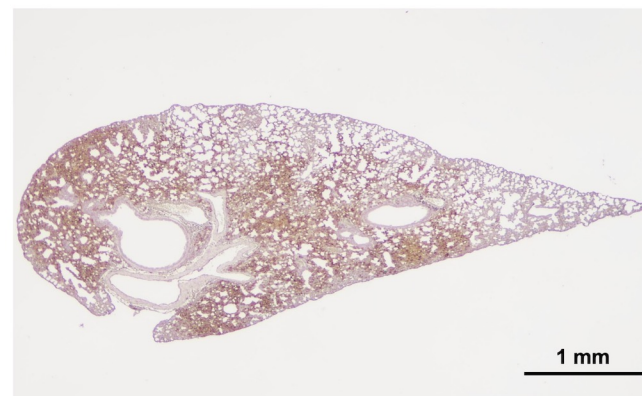
(C)

IHC

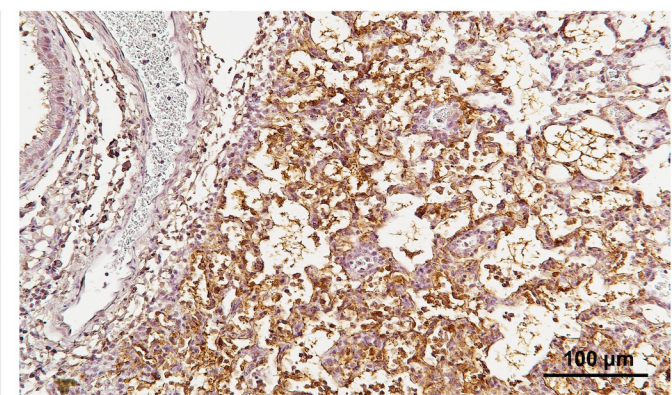
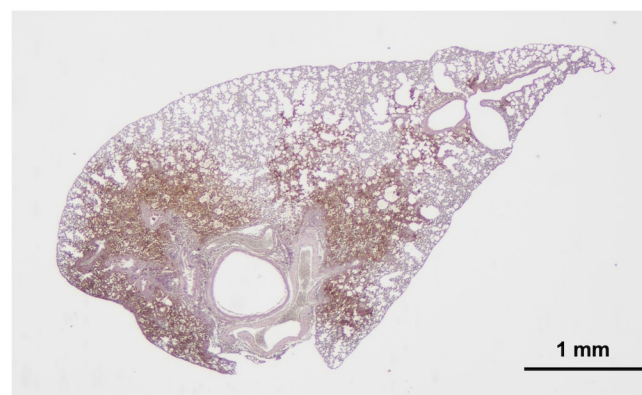
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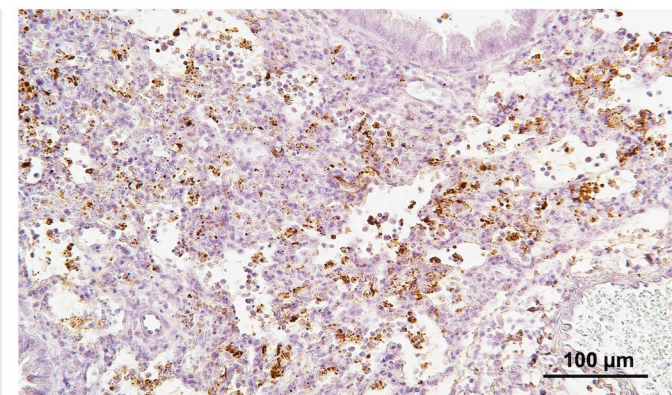
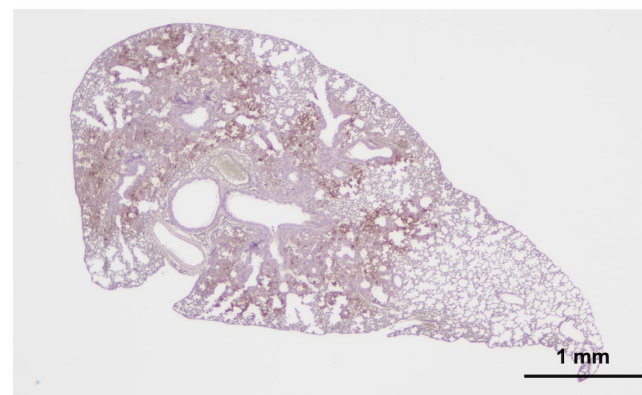
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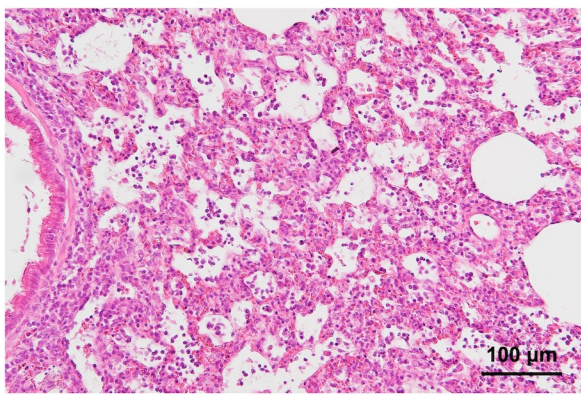


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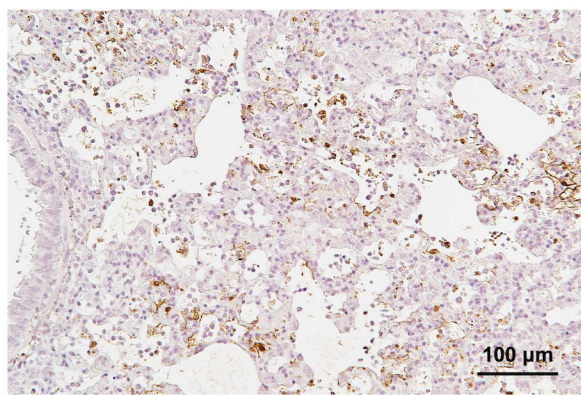


(D)

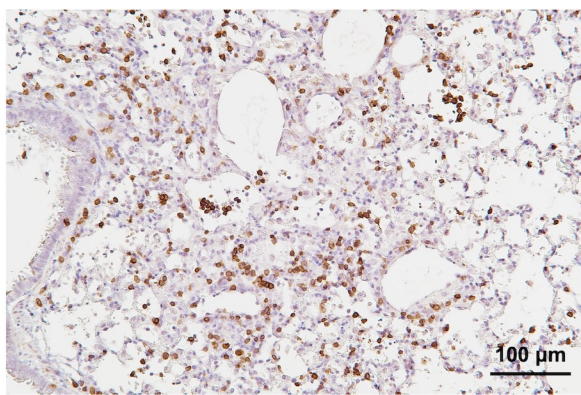
H&E



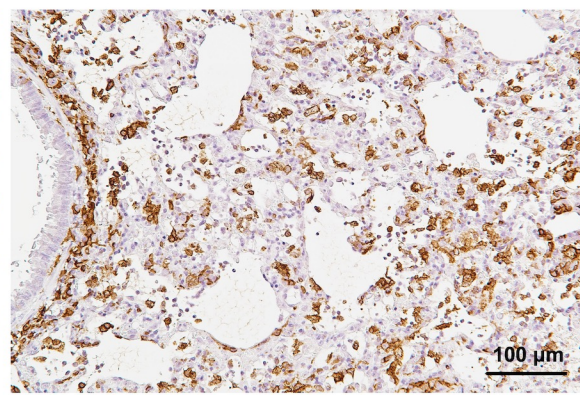
MRV12-52



CD3



Iba 1



MPO

