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ABSTRACT

The coronavirus disease 19 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in unprecedented challenges to healthcare worldwide. In particular, the anthroponotic transmission of human coronaviruses has become a common concern among pet owners. Here, we experimentally inoculated beagle dogs with SARS-CoV-2 or Middle East respiratory syndrome (MERS)-CoV to compare the viral susceptibility and pathogenicity. The dogs exhibited weight loss and increased body temperature and shed the viruses in nasal secretion, faeces, and urine. Mild interstitial pneumonia lesions were observed in the lung tissues of infected dogs. Additionally, clinical characteristics of SARS-CoV-2 infection, such as increased lactate dehydrogenase levels was observed in the current study.

Keywords: SARS-CoV-2, MERS-CoV, dog, pathogenicity

Introduction

Coronavirus disease-2019 (COVID-19) first emerged in China and quickly became a worldwide pandemic. Although most studies have focused on focused on the pathogenesis of COVID-19 in humans, the zoonotic aspects of severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) have raised public health concerns worldwide. In particular, pet dogs living with patients affected by COVID-19 have become infected and shown seroconversion in Hong Kong; these initial cases of human-to-animal transmission of SARS-CoV-2 have suggested potential anthroponotic issues related to COVID-19 [1]. Middle East respiratory syndrome (MERS)-CoV, first identified in Saudi Arabia in 2012, belongs to the beta coronavirus genus, as dose SARS-CoV-2, and has a fatality rate of 34.5% owing to severe respiratory illness [2]. Non-human primates, hDPP4-expressing transgenic mice, and dromedary camels, the natural host of the virus, have been shown to be appropriate animal models for MERS-CoV infection through experimental studies using a variety of animal species [3]. However, no studies have assessed whether MERS-CoV can infect dogs. For SARS-CoV-2, several attempts have been made to select appropriate animal models that accurately recapitulate clinical manifestations of COVID-19 [4]. Dogs have also been inoculated with SARS-CoV-2, and limited pathogenesis, such as viral RNA detection in rectal swabs, was observed [5,6]. Because pet dogs share living space with humans and are a major companion animal as well as an important large animal model for drug development, further analyses are needed to fully establish whether SARS-CoV-2 and MERS-CoV can infect dogs. Accordingly, in this study, we assessed the susceptibility of dogs to SARS-CoV-2 and MERS-CoV following experimental inoculation.

Results

Beagle dogs (9 months old) were experimentally inoculated with SARS-CoV-2 or MERS-CoV, and their susceptibility to these human CoVs was assessed. Most dogs, except for 1 dog inoculated with SARS-CoV-2, showed elevated body temperature compared with the non-infected dog (Figure. 1, A). Weight loss was also observed in most dogs until 7 days-post-inoculation (dpi), whereas the weight of 1 dog infected with SARS-CoV-2 was recovered after 6 dpi (Figure. 1, B).

Next, blood biochemistry and haematological parameters were examined in dogs inoculated with SARS-CoV-2 or MERS-CoV and compared with that in an uninfected dog. Alanine aminotransferase (ALT), albumin (ALB), total bilirubin (TBIL), blood urea nitrogen (BUN), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and creatinine (CREA), white blood cell (WBC) count, lymphocytes (LYMs), monocytes (MONOs), granulocytes (GRANs), haemoglobin (HGB), haematocrit (HCT), platelet (PLT) count, and red blood cell (RBC) count were tested using plasma and whole-blood collected on 0, 3, 5, 6, and 7 dpi. ALT, ALB, BUN, ALP, WBC, LYM, GRAN, HCT, and RBC were within normal range for each parameter. In contrast, some dogs inoculated with SARS-CoV-2 or MERS-CoV showed decreased CREA and PLT levels to less than normal range. Circulating PLT counts are frequently decreased following virus infections, although the mechanisms of PLT/virus interaction are multifaceted [7]. In the clinical setting, coagulation disorders related to lower PLT counts can affect disease severity in patients with COVID-19 and in SARS-CoV- and MERS-CoV- infected patients [8]. In the current study, PLT counts of non-infected dogs were between 342 and 391 $\times 10^3/\mu L$ (normal range: 200-500 $\times 10^3/\mu L$), whereas lower counts were observed in SARS-CoV-2-infected dogs (228, 76, and

$28 \times 10^3/\mu L$ on 3, 5, and 7 dpi, respectively) and MERS-CoV infected dogs (98, 150, and $75 \times 10^3/\mu L$ on 3, 5, and 6 dpi, respectively; Figure. 1, C). MONO counts and HGB levels were somewhat increased in 1 or 2 infected dogs. TBIL levels were elevated in some infected dogs compared with those in non-infected dogs. Consistent with this, increased serum bilirubin levels caused by liver injury were detected in patients with COVID-19 and MERS-CoV infection [9].

LDH is a biomarker that is present in most body tissues and is elevated following tissue damage. Recently, several clinical studies demonstrated that increased LDH levels were associated with disease severity in patients with COVID-19, suggesting that this parameter may be a useful biomarker for disease progression[10-12] . In the current study of experimental infection, LDH levels were significantly increased in dogs infected with SARS-CoV-2 or MERS-CoV; indeed, LDH levels were increased by 1.7-fold at 3 dpi, 4.2-fold at 5 dpi, and 5.5-fold at 6 dpi in three SARS-CoV-2 infected dogs and 2.4-, 4.9-, and 4.1-fold higher at 3 dpi in three MERS-CoV infected dogs compared with those in the non-infected dog (normal range: 40-400 U/L; Figure. 1, D).

Viral RNA levels of SARS-CoV-2 and MERS-CoV in the serum, tonsil, nasal swab, rectal swab, and urethral swab samples of the dogs were tested by conventional real-time polymerase chain reaction kits (Table 1). In the three dogs infected with SARS-CoV-2, viral RNA was detected in nasal swabs, rectal swabs, and urethral swabs at 3, 5, 6, and 7 dpi, whereas MERS-CoV RNA was partially detected in nasal swabs (3 of 3 dogs at 3 dpi, 2 of 3 dogs at 5 dpi, and 2 of 3 dogs at 6 dpi), rectal swabs (2 of 3 dogs at 3 dpi), and urethral swabs (2 of 3 dogs at 3 dpi). All samples were inoculated into Vero E6 cells for analysis of viral viability. SARS-CoV-2 was cultivated from some nasal swabs (3 of 3 dogs at 3 dpi, 2 of 3 dogs at 5 dpi, and 2 of 3 dogs at 6 dpi) and urethral swabs (1 of 3 dogs at 3, 5, and 6 dpi and 2 of 3 dogs at 7 dpi), but not from

rectal swabs. MERS-CoV was not cultivated from any samples. A neutralizing antibody was measured using serum samples, and only one dog infected with SARS-CoV-2 showed an antibody response at 6 dpi (2 Log_2) and 7 dpi (3 Log_2).

Pathological examinations were performed on euthanized dogs at the end of the experiment. On autopsy of dogs infected with SARS-CoV-2, pulmonary consolidation was observed on each lung surface (Figure 2A and 2B), however the other organs were normal. There were no pathologic changes on the organs of MERS-CoV infected dogs. Histopathology from the lung, pharynx, lymph node, spleen, and kidney from all dogs revealed pathologic changes only in lung tissues. Dogs infected with SARS-CoV-2 or MERS-CoV showed similar interstitial pneumonia with mild multifocal peribronchial and perivascular infiltration by inflammatory cells (Figure 2C - 2F). Immunohistochemistry revealed the presence of the SARS-CoV-2 antigen, following viral infection, in the lung and alveolar wall (Figure 2G and 2H), however the MERS-CoV antigen was not detected (Figure 2I).

Discussion

Dogs were first used as a model animal for SARS-CoV-2 by a Chinese research group. Their results showed that dogs exhibited low susceptibility to intranasal inoculation with the virus, as demonstrated by partial virus shedding and no viral detection in tissues [5]. However, we speculated that their data could have been somewhat limited with regard to determining the susceptibility of dogs to SARS-CoV-2. In addition, most pet owners care for their pets a lot and are concerned with the potential for their pets to be infected [13,14].

Therefore, in this carefully designed study, we aimed to demonstrate the pathogenicity of SARS-CoV-2 in dogs based on evaluation of multiple parameters, including clinical signs and blood parameters, and to compare the results with those from another human coronavirus, MERS-CoV. Interestingly, our results were not consistent with the previous study and showed that the dogs exhibited clinical signs of infection after inoculation with SARS-CoV-2 or MERS-CoV. Pathological examinations showed that both human CoVs developed mild interstitial pneumoniae in dogs. SARS-CoV-2 RNA was detected in all nasal and rectal swab samples in this study, and viral viability was even observed in some nasal swabs, whereas viral RNA was not detected in any of the oropharyngeal swabs and in only a few rectal swabs (2 of 5 dogs at 2 dpi, 1 of 4 dogs at 6 dpi, respectively) in a previous experiment [5,6]. Furthermore, SARS-CoV-2 RNA was detected all urethral swabs, and some of the collected viruses could be cultivated. SARS-CoV-2 viability was also detected in urine from a patient with COVID-19 and was confirmed in a clinical study [15]. Among the blood parameters, LDH levels were markedly altered in the dogs in our study. In a recent report, a predictive model using machine learning algorithms and abundant epidemiological, clinical, and laboratory information was established to identify prognostic biomarkers for patients with COVID-19 [16]. The model identified three key features, including LDH levels, as important factors for prognostic prediction in patients with COVID-19 [16]. Consistent with this, in our study, LDH levels were the most prominent parameter affected by respiratory viral infection.

In summary, we experimentally inoculated beagle dogs with SARS-CoV-2 or MERS-CoV and evaluated pathological and clinical changes. Viral infection altered clinical signs and pathology, and viral replication was detected with mild lesions in both infection groups. Experimental infection of the canine species with MERS-CoV was performed for the first time in

the current study. Interestingly, LDH was shown to be a useful follow-up parameter or disease predictive parameter in both SARS-CoV-2 infection and MERS-CoV infection. Overall, our data showed that dogs were susceptible to both human coronaviruses and that they may be feasible as another animal model for SARS-CoV-2 research.

Methods

Viruses

SARS-CoV-2 (NCCP43326) and MERS-CoV (National Control Number 1-001-MER-IS-2015001) were provided by the National Culture Collection for Pathogens in Korea and the Korea Disease Control and Prevention Agency, respectively. The viruses were propagated within Vero E6 cells in Dulbecco's modified Eagle medium (DMEM) with 2% (v/v) foetal bovine serum (FBS), penicillin (10,000 units/mL), streptomycin (10mg/mL), and amphotericin B (25μg/mL) at 37°C in a humidified CO2 incubator. The viral procedures were performed in the Biosafety Level-3 (BL-3) facility of the Korea Zoonosis Research Institute.

Animal studies

All experiments were performed at the Animal Use Biosafety Level-3 (ABL-3) facility at the Korea Zoonosis Research Institute, which is certified by the Korea Disease Control and Prevention Agency of the Ministry of Health and Welfare (certification number KCDC-15-3-02). The animal experiments were conducted in compliance with the ARRIVE guidelines and in accordance with the regulations of the care and use of laboratory animal guidelines of Jeonbuk National University and were approved by the Institutional Animal Care and Use Committee and the experimental protocols requiring biosafety were approved by the Institutional Biosafety

Committee of Jeonbuk National University (approval number JBNU 2020–03-001). Nine-month old female beagle dogs were used for this study. Three randomly selected dogs were anesthetized with Zoletil 50 and were inoculated intranasally with SARS-CoV-2 at a dose of $10^{5.5}TCID_{50}$/mL in 1mL DMEM. Three other dogs were anesthetized and inoculated intranasally with MERS-CoV at a dose of $10^{5.5}TCID_{50}$/mL in 1mL DMEM. One dog was administrated 1mL DMEM intranasally as a negative control. All animals were housed separately in single cages, and clinical signs and body temperatures were monitored for 7 consecutive days post-inoculation (dpi).

Blood biochemistry and haematological examination

For bleeding, dogs were sedated using an intramuscular injection of medetomidine (0.7μg/kg, Tomidin®, Provet Veterinary Products Ltd., Istanbul, Turkey), and blood samples were collected by jugular vein puncture at 0, 3, 5, 6, and 7 dpi. Concentrations of ALT, ALB, TBIL, BUN, ALP, LDH, and CREA were measured in each blood plasma sample using an automated blood chemistry analyser (VetTest 8008, IDEXX Laboratories Inc., Westbrook, ME, USA).

Complete blood counts were determined using an automated haematological analyser (Exigo EOS Vet, Boule Medical AB, Spanga, Sweden). WBC count, LYMs, MONOs, GRANs, PLT count, HGB, HCT, and RBC count were measured using whole blood samples.

Histopathology

All dogs were euthanized using an intravenous injection of 0.1 mg/kg pancuronium bromide and 0.1 M KCl at the end of the experiment (7 dpi). At necropsy, gross lesions were observed seen mostly in the lung, pharynx, lymph nodes, spleen, and kidney, and then tissues were

collected and fixed in 4% neutral-buffered formalin for 1 week. Tissues embedded in paraffin blocks were sectioned at a thickness of 4 μm and then mounted onto glass slides. The slides were deparaffinized in xylene and rehydrated through a series of graded 100% ethanol to distilled water and then stained with haematoxylin and eosin. For immunohistochemistry to detect the SARS-CoV-2 antigen, the deparaffinized and rehydrated slides were blocked for endogenous peroxidase with 3% H_2O_2 in phosphate buffered saline (PBS) for 20 min. The tissue sections were placed in 10 mM citrate buffer (pH 6.0), heated for 1 h, and incubated with SARS Nucleocapsid Protein Antibody (NB100-56576, Novus, USA) at a 1:200 dilution at 4 ℃ overnight. For MERS-CoV antigen detection, the tissue sections were digested with proteinase K (P2308, Merck, Germany) for 30 min at 37 ℃ and incubated with rabbit polyclonal antiserum against MERS-CoV (Sino Biologicals Inc., China) at a 1:1000 dilution at 4 ℃ overnight. All slides were then washed in PBS and incubated with the secondary antibody (RealTM EnvisionTM Detecion system rabbit/mouse, K5007, Dako, Denmark) for 40 min at 37 ℃. Colour development was performed using 3, 3'-diamino-benzidine tetrahydrochloride (DAB; K5007, Dako, Denmark) followed by counterstaining with haematoxylin. Light microscopic examination was performed using a BX53 microscope (Olympus, Japan).

Quantitative real-time PCR

Viral loads were analysed by quantitative real-time PCR using commercial one-step real-time PCR kits for SARS-CoV-2 (Allplex 2019-nCoV Assay kit, Seegene, Seoul, South Korea) and MERS-CoV (PowerChek MERS Real-time PCR Kit, Kogenebiotech, Seoul, South Korea). Tissue samples from all dogs were placed into soft tissue homogenizing CK14 tubes (Precellys, Betin Technologies) prefilled with ceramic beads and DMEM and then homogenized using a

Bead blaster 24 (Benchmark Scientific, NJ, USA). Viral RNA was extracted from the homogenized tissues using the QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. Real-time PCR for each virus was conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

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Author contributions statement

K.S.L., J.Y.L., K.C.K., and D.S. designated this study and discussed the results. Y.Y., S.G.L., M.Y., and E.H.B. performed experiments and analyzed data. K.S.L. and D.S. wrote the manuscript.

Competing interests

Authors declare no competing interests.

Table 1. Viral RNA detection and virus cultivation of SARS-CoV-2 or MERS-CoV in tissue samples.

		Lung	Tonsil	Serum					Nasal					Anal					Urethra				
				dpi [a]					dpi					dpi					dpi				
				0	3	5	6	7	0	3	5	6	7	0	3	5	6	7	0	3	5	6	7
SARS-	A	-	-	-	-	-	-	-	-	+[b](+)[c]	+(+)	+(+)	+	-	+	+	+	+	-	+	+	+	+
CoV-2	B	-	-	-	-	-	-	-	-	+(+)	+	+(+)	+	-	+	+	+	+	-	+	+	+	+(+)
	C	-	-	-	-	-	-	-	-	+(+)	+(+)	+	+	-	+	+	+	+	-	+(+)	+(+)	+(+)	+(+)
MERS-	A	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-
CoV	B	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	+	-	-	-
	C	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Cont.		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Viral RNA detection by a commercial real-time PCR kit,

[b] dpi: days-post-inoculation,

[c] ( ): virus cultivation in Vero-E6 cells

Figure. 1: Body temperature changes, weight loss, platelet (PLT) count, and lactate dehydrogenase (LDH) levels of dogs inoculated with SARS-CoV-2 or MERS-CoV. Three beagle dogs in each group were inoculated intra-nasally with SARS-CoV-2 ($10^{5.5}$ $TCID_{50}$/mL) or MERS-CoV ($10^{5.5}$ $TCID_{50}$/mL). Temperature change (A), weight loss (B), PLT count (C), and LDH levels (D) were measured.

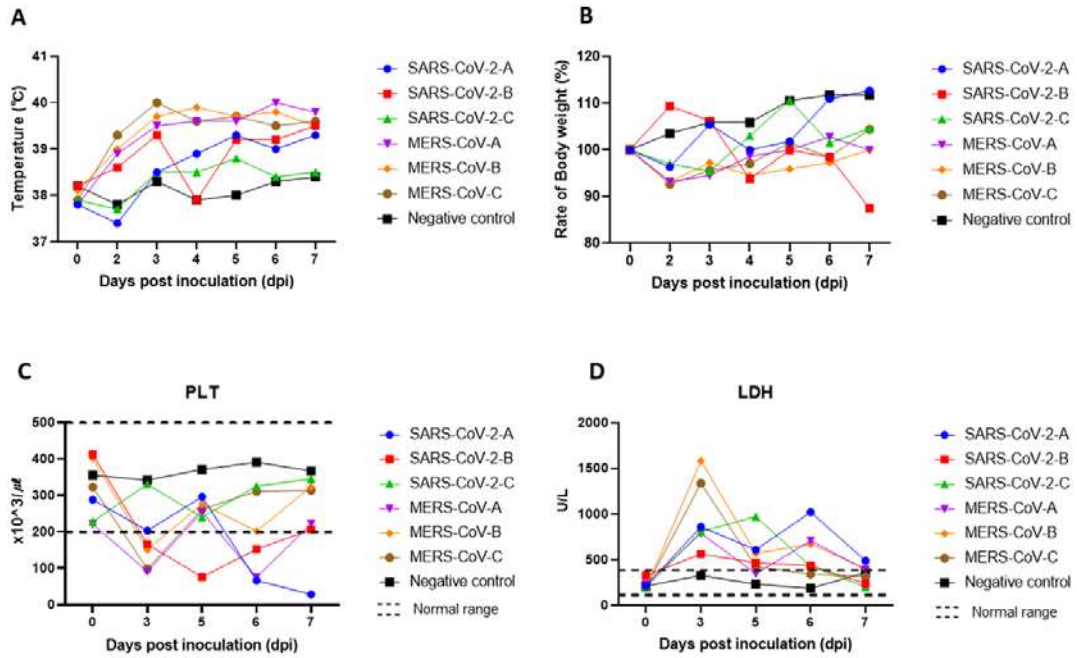
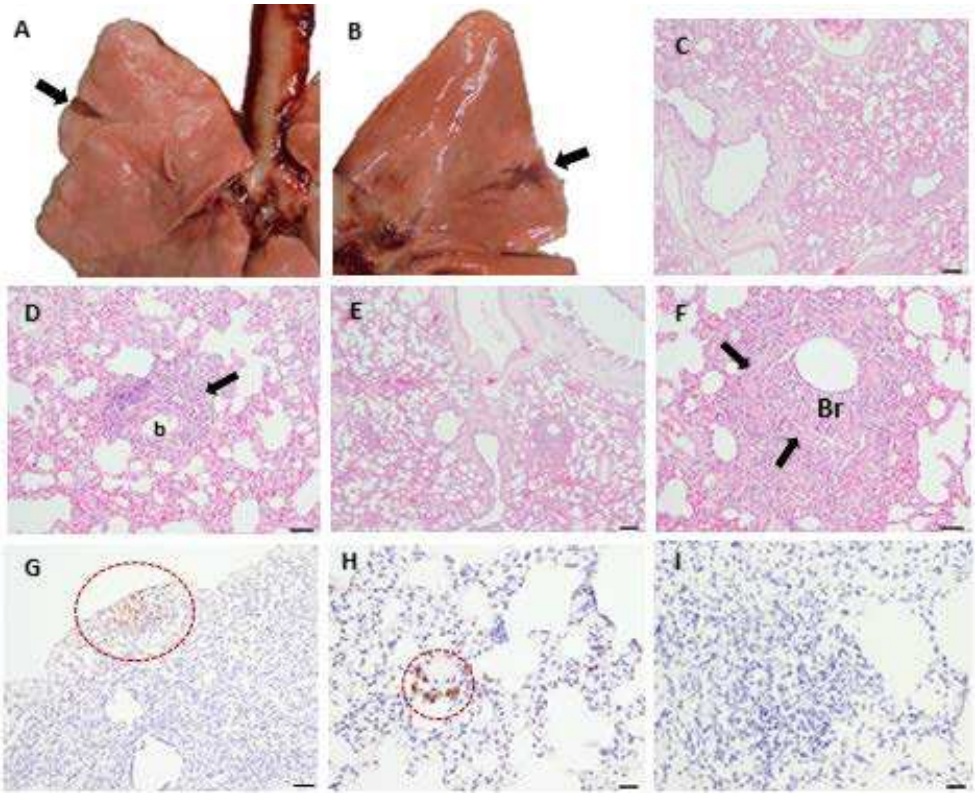


Figure. 2: Pathologic changes in lungs of dogs inoculated with SARS-CoV-2 or MERS-CoV. Pulmonary consolidation (arrowhead) in right dorsal lobe of a SARS-CoV-2 infected dog (A) and in left dorsal lobe of another SARS-CoV-2 infected dog (B). Histological change showed mild interstitial pneumonia in a SARS-CoV-2 infected dog (C; x40, haematoxylin and eosin stained) and perivascular inflammatory cell infiltration (arrowhead) was determined (D; x200, b: blood vessel). Mild interstitial pneumonia was observed in a MERS-CoV infected dog (E; x40). Focal bronchiolitis (Br) lesion with perivascular inflammatory cell infiltration (arrowhead) was determined (F; x200). SARS-CoV-2 antigen detection by immunohistochemistry (IHC) in lung (G; x100; bar: 50µm) and alveolar wall (H; x200; bar: 20µm). MERS-CoV antigen was detected by IHC (I; x100; bar: 50µm)



Figures

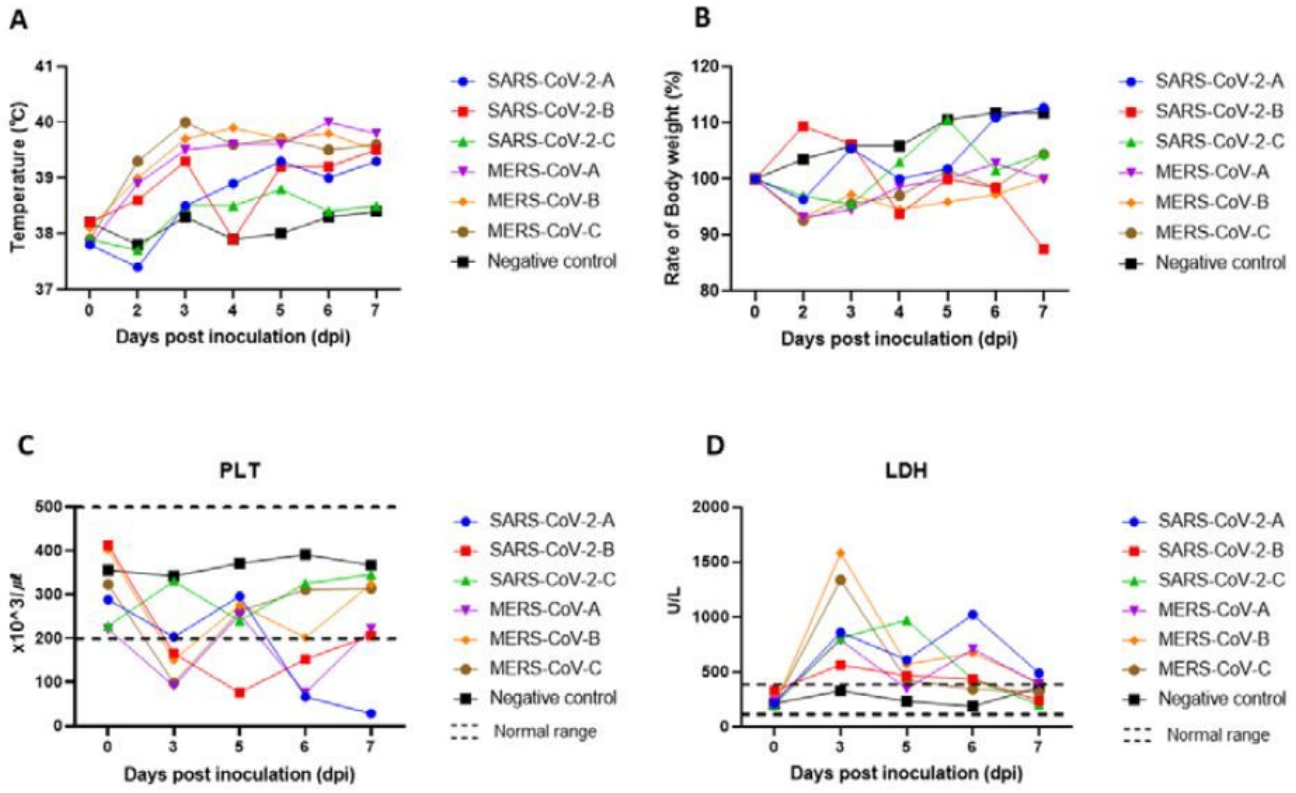


Figure 1

Body temperature changes, weight loss, platelet (PLT) count, and lactate dehydrogenase (LDH) levels of dogs inoculated with SARS-CoV-2 or MERS-CoV. Three beagle dogs in each group were inoculated intranasally with SARS-CoV-2 (105.5 TCID50/mL) or MERS-CoV (105.5 TCID50/mL). Temperature change (A), weight loss (B), PLT count (C), and LDH levels (D) were measured.

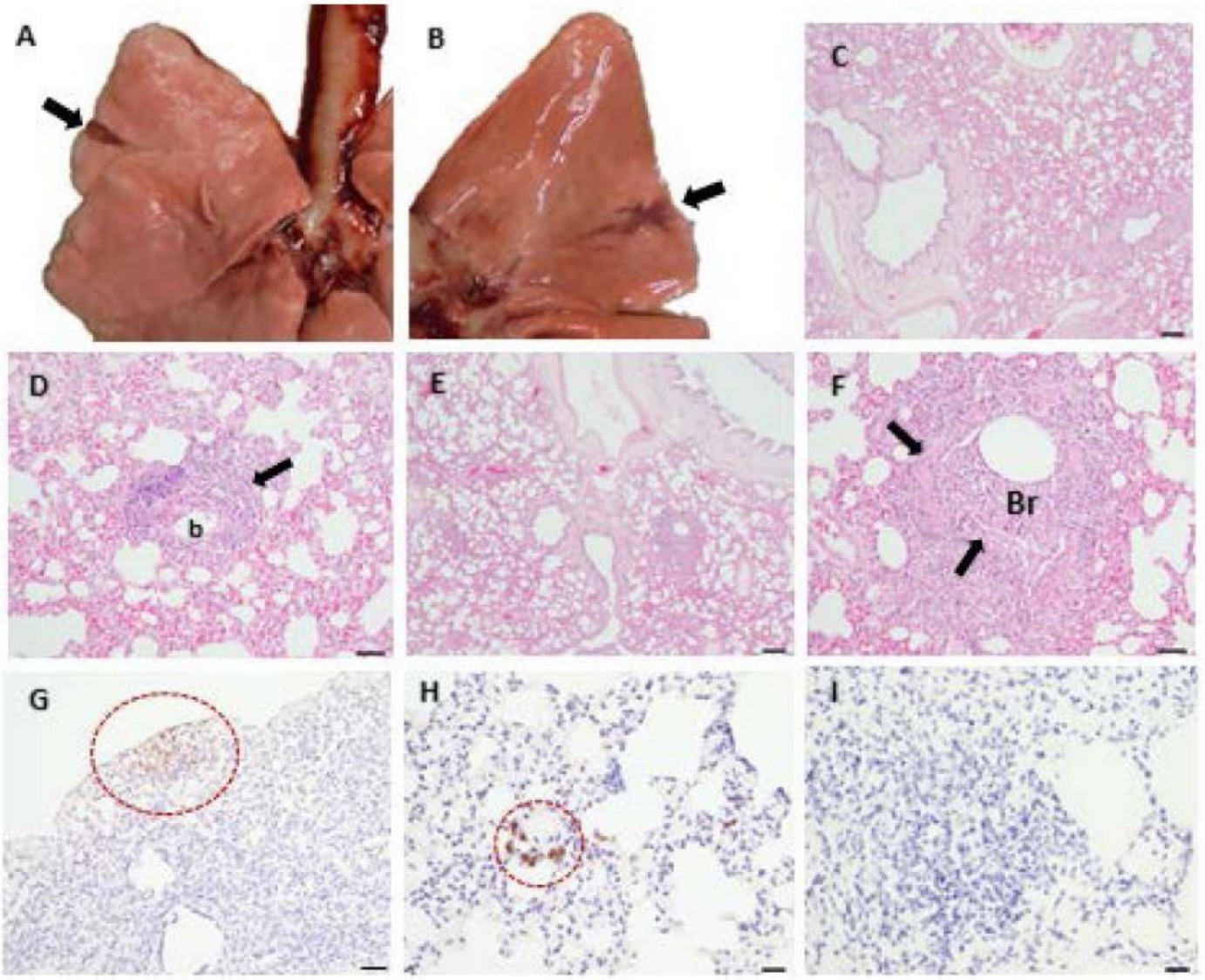


Figure 2

Pathologic changes in lungs of dogs inoculated with SARS-CoV-2 or MERS-CoV. Pulmonary consolidation (arrowhead) in right dorsal lobe of a SARS-CoV-2 infected dog (A) and in left dorsal lobe of another SARS-CoV-2 infected dog (B). Histological change showed mild interstitial pneumonia in a SARS-CoV-2 infected dog (C; x40, haematoxylin and eosin stained) and perivascular inflammatory cell infiltration (arrowhead) was determined (D; x200, b: blood vessel). Mild interstitial pneumonia was observed in a MERS-CoV infected dog (E; x40). Focal bronchiolitis (Br) lesion with perivascular inflammatory cell infiltration (arrowhead) was determined (F; x200). SARS-CoV-2 antigen detection by immunohistochemistry (IHC) in lung (G; x100; bar: 50μm) and alveolar wall (H; x200; bar: 20μm). MERS-CoV antigen was detected by IHC (I; x100; bar: 50μm)

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