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Research Article

Comparison of Mouse Species in an *In Vivo* SARS-CoV-2 Challenge Model

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ABSTRACT

The K18-hACE2 transgenic mice, a model animal having human ACE receptors, is employed in studies against the SARS-CoV-2 virus all over the world. Aged Balb/C mice utilized during the SARS-CoV outbreak were compared to non-T-cell, immunosuppressive Nude mice often employed in cancer research and K18-hACE2 transgenic mice used as a model animal against the SARS-CoV-2 virus challenge assay. At the same time, the role of the model animal K18-hACE2 transgenic mice in organs other than the lung was studied. The BSL3 facility was used for the challenge experiment in this study. In three groups, 105 TCID50 SARS-CoV-2 virus B.1.1.7 (the alpha variant was gavaged and intranasally administered to mice under anesthesia. The experiment was ended on the tenth day, and gross pathology was done. The viral load of SARS-CoV-2 was determined by RT-PCR after collecting the target organ lungs from all mice as well as the spleen, liver, heart, and kidneys from the K18-hACE2 transgenic mouse group. In comparison to Balb/C and Nude mice, the K18-hACE2 transgenic mouse model animal has been shown to be a suitable model against the SARS-CoV-2 virus in our study. At the same time, when the organs of K18-hACE2 transgenic mice were compared, viral load retention occurred in the target organ, the lung, with no significant retention in other organs.

Keywords: COVID-19, *In vivo* challenge, Model animals, SARS-CoV-2

Introduction

The SARS-CoV-2 virus, which infected 767 million individuals and killed 6.9 million people globally, was identified as the causal agent of COVID-19 [1-4]. Coronaviruses (CoVs) are members of the Coronaviridae family, the Nidovirales order, and the genus Coronavirus. Coronaviridae, the biggest group of viruses, is divided into two subfamilies: Coronavirinae and Torovirina. Coronavirinae is further subdivided into four generations: alpha, beta, gamma, and delta coronaviruses [5-8]. SARS-CoV, a coronavirus that emerged in 2002-2003 with a 10% mortality rate, manifested itself as a lethal disease that caused severe acute respiratory syndrome (ARDS). Middle East respiratory syndrome coronavirus (MERS-CoV), discovered in Saudi Arabia nearly a decade later, caused similar devastation and loss, with a 35% mortality rate. SARS-CoV-2, a third member of the Coronaviridae subfamily, emerged as a new deadly disease in December 2019 and was declared a pandemic by the World Health Organization [3,9].

Humans with SARS-CoV-2 infection have developed a variety of diseases, some of which are asymptomatic and occasionally show serious symptoms. Severe COVID-19 symptoms typically include progressive respiratory failure that necessitates hospitalization and ventilation. The disease's lethal state has been caused by ARDS, which is associated with inflammation and thrombosis, often resulting in multiple organ failure [10,11]. Epidemiological studies have shown that age, gender, diabetes, and obesity are all risk factors for the development of severe COVID-19 [2,12].

Based on rapidly evolving data, the National Institutes of Health (NIH) and the Infectious Diseases Society of America (IDSA) have developed the most recent COVID-19 Treatment Guidelines [13]. More than 40 vaccines, hyperimmune serums, numerous drugs, and therapeutic molecules are being studied in clinical trials, with another 150 being studied in preclinical studies [14]. Before entering the clinical stage, the final efficacy of all types of protective and therapeutic products is evaluated with an *in vivo* challenge test. Although different animal models are used in COVID-19 *in vivo* studies, mice are the most preferred model in terms of accessibility and cost. Model animal studies for Corona viruses began during the SARS-CoV and MERS-CoV epidemics. Different

mouse breeds (Balb/C, C57BL/6, B6, and 129S) have been investigated in these diseases, but the expected response could not be obtained. Balb/C mice vaccinated with SARS-CoV showed no clinical symptoms and some virus recovery, despite gaining weight. Although viral RNA was found in the lungs and intestines of these mice, there was no mortality [15,16]. SARS-CoV studies employed Balb/C mice at 21 weeks of age to capture clinical signs [17-19]. In MERS CoV experiments, however, it was found that aged Balb/C mice did not exhibit appropriate clinical signs, and the virus titer remained low. Because of the incompatibility of spike (S) protein with mouse ACE2, conventional laboratory mice did not support MERS-CoV, SARS-CoV, and SARS-CoV-2 infections, which have entered our lives. As a result, a new transgenic mouse model has been developed to replicate human disease as well as for pathogenesis investigations and the development of antiviral treatments [2,16,19,20]. K18-hACE2 transgenic mice may express the human ACE2 receptor, which is utilized by SARS-CoV-2 [21,22]. The hACE gene is expressed in epithelial cells in these transgenic mice under the control of the cytokeratin 18 promoter [23,24]. The development of model mice distinct from Balb/C and C57BL/6 mice has been critical for understanding the mechanisms of SARS-CoV-2 and developing treatment strategies.

K18-hACE2 transgenic mice are produced and sold at few sites around the world. However, many experimental animal facilities that desire to conduct SARS-CoV-2 research do not have this mouse strain. In this study, the Balb/C mouse and Nude mouse strains, both of which are widely available in Türkiye, were compared to K18-hACE2 transgenic mouse strains against the SARS-CoV-2 virus. The Balb/C mouse research carried out during the SARS and MERS CoV outbreaks was replicated in this investigation under current settings. Another aim of our research was to compare the quantity of virus uptake in various organs and the lung of a SARS-CoV-2 virus model animal, the K18-hACE2 transgenic mouse.

MATERIAL AND METHODS

Ethics Statement

All experimental procedures with animals were approved by TUBITAK (Marmara Research Center) MRC, Life Sciences, Medical Biotechnology Unit in Kocaeli, TÜRKİYE. All procedures in this study involving animals were reviewed and approved by the Institutional Biosafety Committee and Institutional Animal Care and Use Committee (HADYEK-16563500-111-3026); all the experiments were conducted in compliance with all relevant ethical regulations. The experiments were conducted in BSL3 and animal BSL3 (ABSL3) facilities at TUBITAK MRC Life Sciences.

Animals

The transgenic K18-hACE2 [B6.Cg-Tg(K18-hACE2) 2Prlmn/J] mice used in this study were provided by the Jackson Laboratory in the United States. TUBITAK MRC Life Sciences Experimental Animals Unit has rights for the production of K18-hACE2 transgenic mice. All of the experiments were conducted in a biocontainment isocage, which is part of ABSL 3. 16-18 week-old female Balb/C and Nude mice and 8-10 week-old female K18-hACE2 transgenic mice were used, and 4 mice (20-30 g body weight) were used for each group. The animals kept in an environment with a temperature of 20-24°C, with a controlled light cycle (12 h light and 12 h dark) and consumption of SPF solid food and water ad libitum throughout the experimental period. There were three groups in this study: the Balb/C mice group, the nude mice group, and the K18-hACE2 transgenic mice group.

Challenge Method for SARS-CoV-2 Infection in Mouse Models

SARS-CoV-2 virus strain B.1.1.7 (alpha variant) was isolated from patients by the Ministry of Health Directorate of Public Health and provided to us for this study. All virus growth was done in biosafety level 3 (BSL-3) labs at the TUBITAK Marmara Research Center (TUBITAK MRC), Life Sciences, and Medical Biotechnology Unit in Kocaeli, TÜRKİYE. This laboratory had all the international certificates needed to work with SARS-CoV-2.

In the BSL 3 facility, Balb/C, Nude, and K18-hACE2 mice were housed in biocontainment cages for a challenge experiment with the SARS-CoV-2 virus B.1.1.7 with a $TCID_{50}$ value of 10^5. It was kept in the laboratory for two days for adaptation to the environment. In the study's model, $1x10^5$ $TCID_{50}$ virus was administered to the animals via gavage and intranasal administration. For the first three days of the experiment, the animals were given SARS-CoV-2 virus orally via gavage, followed by 50 µL of 10^5 $TCID_{50}$ intranasally under anesthesia *(Fig. 1)*. The mice's gavage dosage was modified according to their

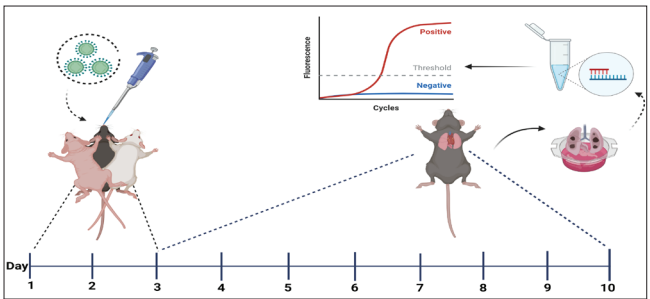

Fig 1. Representation schematic of the *in vivo* challenge experiment. Balb/C, Nude, and K18 hACE2 mice were administered the SARS-CoV-2 (Alpha) virus for three days. On the tenth day of the experiment, autopsy-collected lungs were analyzed by PCR to ascertain the amount of viral RNA (n = 4/group)

weight, however the maximum quantity they may aspirate with intranasal administration is 50 µL. As a result, all mice received the same doses of virus intranasally. The experiment lasted a total of ten days. After being infected with the virus, mice were monitored on a daily basis for morbidity (body weight) and mortality. Mice that lost more than 25% of their baseline body weight were considered to have reached the experimental endpoint and were exterminated. Pathological examinations were performed after the animals were sacrificed. All abdominal organs and thoracic cavity organs were examined with the naked eye during gross pathology. Each mouse lung was used to compare viral load by real-time PCR analysis [25-27]. During gross pathology, samples were taken to compare how much of the SARS-CoV-2 virus was absorbed into the lung, heart, spleen, liver, and kidneys of K18-hACE2 transgenic mice.

The lungs were evaluated and scored with the naked eye in terms of color, tissue integrity, appearance, and size during gross pathology. Organs that were clean and free of lesions received a score of 0 (zero), while organs with lesions such as edema, hyperemia, and pneumonia received a score ranging from 1 to 5 (one to five) [28].

Tissue Homogenization

The mice's lungs and other organs were sonicated for viral RNA analysis. The organ tissues were homogenized separately in 2 mL of PBS using an ultrasonic homogenizer at 70% amplitude for 90 sec (Bandelin HD2200.2, Germany) for viral isolation. Tissue homogenates were centrifuged at 21.500 x g for 10 min, and supernatants were collected into 15-mL falcon tubes.

Viral RNA Isolation and RT-PCR

Viral RNA was extracted with the QIAamp Viral RNA Mini Kit, Cat. No. 52906 (QIAGEN, USA) according to the instructions of the manufacturer. The viral RNA detection was performed using SARS-CoV-2 nucleocapsid-specific primers and probes detailed below with the One Step PrimeScript III RT-PCR Kit (Takara, Japan). All reactions were performed on a CFX96 Touch instrument (BioRad, USA) with the following Real-Time PCR conditions: 52°C for 5 min, 95°C for 10 sec, then 44 cycles of 95°C for 5 sec and 55°C for 30 sec. The primer and probe sequences that are used for RT-PCR are CDC-recommended and FDA-approved (EUA) NC1 and NC2 primer-probe sets whose target region is the Nucleocapsid (NC) gene of SARS-CoV-2. (Primer and probe sequences are: N1 Forward: 5'-GAC CCC AAA ATC AGC GAA AT-3', N1 Reverse: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3, N1 Probe: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3 N2 Forward: 5'-TTA CAA ACA TTG GCC GCA AA-3' N2 Reverse: 5'-GCG CGA CAT TCC GAA GAA-3' N2 Probe: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3) [29]

Statistical Analysis

An unpaired one-way ANOVA was used for comparison between three groups. A two-sided P value <0.05 was considered statistically significant. Statistical analyzes and graphs was performed with GraphPad Prism 5 programs.

Results

Clinical Evaluation

Throughout the study, the weight and clinical symptoms of the mice in all three groups were monitored. K18-hACE2 transgenic mice were the most affected clinically. Weight loss began in virus-infected mice on the fifth day of the trial, and by the end of the experiment, approximately 17-29% live weight reduction was seen. The animals in the nude mouse group had a slight clinical effect. Two out of four mice lost approximately 6.5% of their live weight, whereas the other two mice lost no weight. The Balb/C mouse group was not clinically affected. One mouse lost 3.5% of its body weight, whereas the other mice gained weight *(Fig. 2)*.

Gross Pathology

On the tenth day of the study, the animals in the experiment were sacrificed using the cervical dislocation procedure, and gross pathology was done. Pneumonia was observed in the lungs of two mice in the K18-hACE2 transgenic mouse group, and hemorrhagic regions occurred in the lungs of the remaining two mice, depending on erythrocyte density. Hemorrhagic regions have been found in the lungs of two naked mice and one Balb/C mouse. In Nude and Balb/C mice, no abnormal signs were found in the lungs or other organs of the remaining animals. *Fig. 3-A* shows the gross pathology-related appearance of the animal lungs that were obtained, and *Fig. 3-B* shows the score graph.

Viral Load Assessment by RT-PCR

In this study, the presence of the virus was determined using RT-PCR and viral RNA from the lungs and other

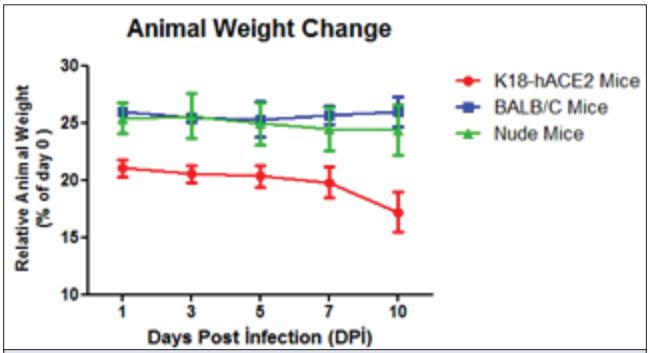

Fig 2. Graph demonstrating the mean change in body weight of challenged mice over a 10-day period. Only the K18 hACE2 group showed a significant response after seven days. One-way ANOVA test was used for statistic (P<0.0001), between-group variation was found to be significant

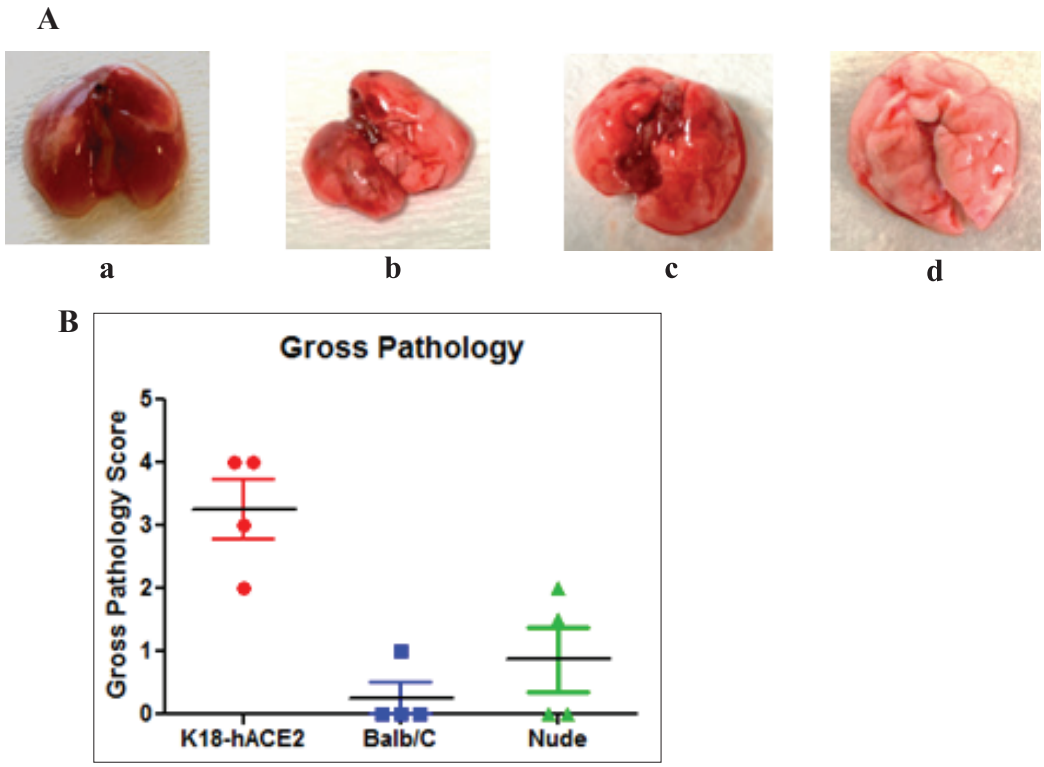

Fig 3. A) Gross pathology images of experimental mice's lungs **a)** Pneumonia-formed lung of K18 hACE2 transgenic mouse; **b)** hyperemic lung of nude mouse; **c)** Less hyperemic lung of Balb/C mouse; and **d)** intact, healthy lung of Balb/C Mouse, **B)** Gross pathological inflammation score graph of lungs from three groups of mice. Lung inflammation was graded on a scale of 0 to 5 (0 for no inflammation, 1 for low-level hyperemia, 2 for significant pathological lesion-hyperemia, and 3-5 for various stages of pneumonia). One-way ANOVA test was used for statistic (P<0.0020), but the findings were determined to be non-significant

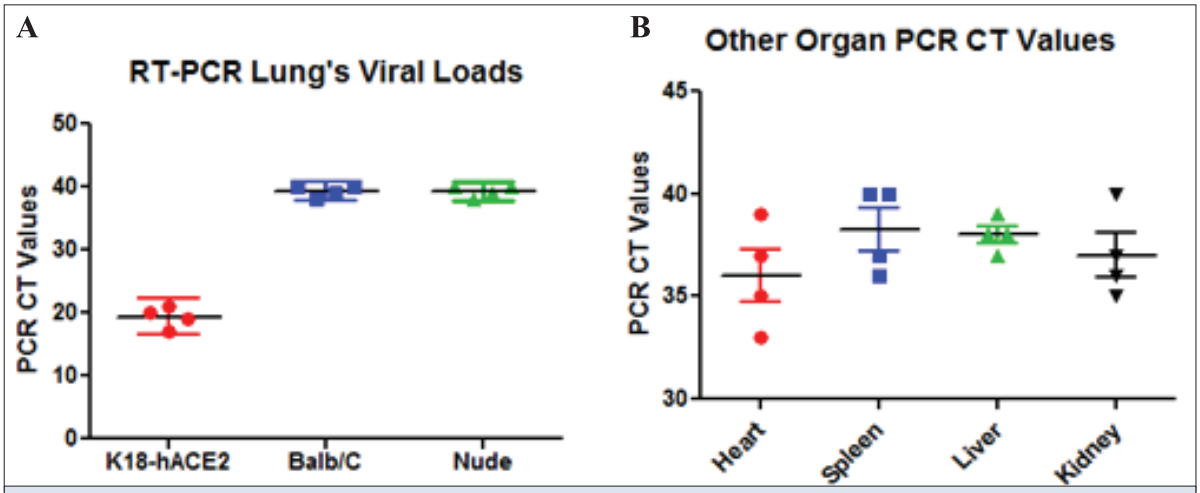

Fig 4. Viral loads in lung and other organs determined by Real Time PCR targeting two distinct regions of the SARS-CoV-2 Nucleocapsid gene. One-way ANOVA test was used for statistics. **A)** Results for the viral loads in the lung were significant (p<0.0001), according to RT-PCR, **B)** Comparing viral loads in other organs, however, was not shown to be significantly distinct (P<0.4096)

organs. CT measured between 16 to 21 in the K18-hACE2 transgenic mice group. CT measured between 37 to 40 in the Nude and Balb/C mice groups. In the K18-hACE2 transgenic group, between 33 to 40 viral CTs were acquired from different organs of mice. The CT averages for these organs are as follows: heart 36%, spleen 38%, liver 38%, and kidney 37% *(Fig.4-A,B)*.

Discussion

Many studies with K18-ACE2 transgenic mouse models

against COVID-19 disease have been published in the literature. According to Winkler et al.[23] following intranasal infections of K18-ACE2 mice at 4 dpi (Day Post İnjection) and 7 dpi, the animals lost significant weight and had high virus loads at 2 dpi, 4 dpi, and 7 dpi. According to this study, the most virus was found in the lungs of hACE2 mice at 3 dpi, followed by a decrease at 5 dpi and a continued decrease at 7 dpi [30]. The results of these studies have demonstrated that if K18-ACE2 transgenic mice are exposed to the virus every other day, the viral load begins to decline after the fifth day, becomes minimal after 7-9 days, and the virus disappears within a few days. According to the literature, we used a 3 dpi for the groups in our study [26,27]. The study contrasted the Balb/C mouse strain, which was used in the past for MERS-CoV and SARS-CoV infections, as well as the immunosuppressed CD1-nude (Foxn1null) mouse race and K18-ACE2 transgenic mice, which are model animals. Furthermore, the heart, spleen, liver, and kidneys of mice in the hACE2 group were collected, and the role of the SARS-CoV-2 virus was investigated [25,30].

When the clinical symptoms of $1x10^5$ B.1.1.7 strains and different breeds of mice were studied in this study, Balb/C mice had no effects at all, and the animals even gained weight at the end of the experiment. At the end of the investigation, naked mice lost about 1.5 g of weight, but no additional clinical signs were observed. K18-ACE2 transgenic mice, on the other hand, experienced significant weight loss as well as clinical signs such as put-off movements, abdominal breathing, stooped posture, and eye burrs.

In their study of K18 hACE2 transgenic mice, Oladunni et al.[21] investigated the histological changes and gross pathology changes of organs such as the lung, brain, liver, and spleen in 2 dpi and 4 dpi groups. They linked neutrophil and lymphocyte infiltration into the alveolar regions to the emergence of slight pneumonia at 2 dpi. At 4 dpi, the pneumonia rate increased fourfold, the alveolar gaps narrowed with hemorrhagic hemorrhages, and inflammatory cell infiltration increased. In terms of gross pathology, there is no significant pathological lesion in the lungs or other organs of Balb/C or Nude mice. Erythrocyte and lymphocyte infiltration of the lungs was seen in one Balb/C mouse and two Nude mice. In four K18-ACE2 transgenic mice, pneumonia was shown to be graded 2-4 in the lungs [28].

In this study, the presence of virus in the lungs was determined by RT-PCR, and viral density was calculated using CT values. CT ranged from 16 to 21 in the K18-hACE2 transgenic mice. Early CT values indicate a large number of viruses in this group of animals. Nude and Balb/C mice, on the other hand, produced 37-40 CT. CTs of 30 or above show that there is very little viral RNA *(Fig.4-A)*. As a result, even though CT values were found in the Nude and Balb/C groups, the results were deemed negative [26-28]. Because there was no viral load in the target organ lungs of Balb/C and Nude mice, only K18-hACE2 transgenic animals were studied for viral uptake in other organs. When comparing CT averages from organs other than the lung, low positive viral results were obtained, as in the studies of Sun et al.[25] and Johansen et al.[16]. According to our results, when we compared the viral involvement of the organs, they were heart > kidney > spleen > liver, respectively *(Fig. 4-B)*.

This investigation compared prior investigations using Balb/C, Nude, and K18-hACE2 transgenic mice groups. Older Balb/C mice employed in this study, on the other hand, displayed a substantially lower, non-significant response to the SARS-CoV-2 virus. This study showed that Balb/C experiments against Coronaviruses are not scientifically sufficient at the present time. But we don't know if this is just tied to the SARS-CoV-2 virus or if it would have responded similarly to the SARS-CoV virus today. It was revealed that CD1 nude mice, an immunosuppressive mouse strain, did not respond to SARS-CoV-2 tries as expected. With this study I demonstrated that only the K18-hACE2 transgenic mouse line should be utilized in COVID-19 mouse model experiments to produce accurate results. Despite the presence of SARS-CoV-2 virus in other organs in this study, necropsy showed that it did not cause clinical damage in these organs due to its low level virus.

Availability of Data and Materials

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

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