1 **History repeats itself: Horse originated Hyperimmune sera production against**
2 **SARS CoV-2**

3 ## Abstract

4 **Background/aim:** SARS-CoV-2 disease was announced as a pandemic by The World
5 Health Organization in early 2020. It is still threatening the world population. Here, we
6 aimed to produce hyperimmune sera that contain immunoglobulin G and F(ab')$_2$
7 fragments sourced from horse antibodies as an urgent response to the pandemic.

8 **Materials and methods:** SARS-CoV-2 was produced and inactivated with three different
9 methods [formaldehyde (FA), formaldehyde, and binary ethylene amine (FA+BEI), and
10 heat treatment]. After *in vitro* inactivation control, immunogens were mixed with
11 Freund's adjuvant, thereafter horses (n: 2 for FA, 4 for FA + BEI, 2 for Heat inactivation)
12 and New Zealand rabbits (n: 6 for FA, 6 for FA + BEI, 6 for Heat inactivation) were
13 immunized four times. Neutralizing antibody levels of the sera were measured at the 4th,
14 6th, and 8th weeks. When the antibodies were detected at the peak level, plasma was
15 collected from horses and hyperimmune sera procured after the purification process.

16 **Results:** Horses and rabbits produced highly neutralizing antibodies against the SARS-
17 CoV-2 in FA and FA + BEI inactivation groups, foreign proteins were removed
18 effectively after purification.

19 **Conclusion:** This study presents a profitable practice to develop horse-specific antisera
20 against SARS-CoV-2 for emergency and low-cost response. In further studies, new
21 purification methods can be used to increase the efficiency of the final product.

22 **Keywords:** Antibody cocktail, F(ab')$_2$, hyperimmune sera, horse sera, immunoglobulin,
23 SARS-CoV-2

24 ## 1. Introduction

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) emerged in late 2019 and has been causing a pandemic since March 2020. Today, there are 178,207,851 confirmed coronavirus cases, and 3,858,077 deaths reported all over the World (https://www.worldometers.info/coronavirus/, 18 June 2021). Although authorities are trying to decrease the spreading of the virus by wearing masks, social distancing, lockdowns, and related regulations, there is still an enormous increase in exposure. Vaccine development as a decisive solution is still in progress and although humanity is on the brink of vaccine production and distribution, many will be dependent on therapeutics until large numbers of people could be vaccinated. Still, treatment algorithms are mainly based on symptom-relieving therapeutics. Different types of drugs have been used in the treatment of COVID-19 since the start of the pandemic [1]. Inventions on treatment and prophylaxis are crucially needed [2].

A healthy immune system is essential for rapid recovery after SARS-CoV-2 exposure. Different types of immunotherapies procured from patients (convalescent), animals, or biotechnology sources are implemented as treatment options as well [3]

Convalescent plasma therapy is a therapeutic and prophylactic treatment based on using the acellular portion of a patient's blood after recovery. This method can be used as a cure for viral diseases or prevention of disease in individuals who are in risk groups [4].

There were reports on the promising results of convalescent plasma therapy in COVID-19 [1, 5, 6]. Convalescent plasma therapy is related the stage of the illnesses and the amount of the administered dose. In the early stages of infection, convalescent plasma containing polyclonal neutralizing antibodies may inhibit viral entry as well as replication [5]. Different doses (200-500 mL / 50-80 kg patient) were administered in to the COVID-19 patients [5,7]. On the other hand, convalescent plasma has some disadvantages; its

production and storage are expensive, it is dependant on patients who recovered from COVID-19, takes time to use, needs to be controlled for the existence of other pathogens, and has limited availability for purification. Production and standardization of large amounts of convalescent plasma is very difficult.

Likewise, the animal-originated hyperimmune serum has also been a treatment choice for diseases. Hyperimmune serum invention goes back to the 1890s, especially its application against diphtheria [8]. The hyperimmune serum was successfully produced in Etlik Veterinary Control Central Research Institute (Etlik VCCRI) and used in the studies during 1921-1930 in Turkey [9].

Animal-derived immunoglobulin such as equine sourced anti-SARS-CoV $F(ab')_2$ was reported to be effective in cell culture and murine and the hamster *in vivo* models [10, 11]. Moreover equine sourced $F(ab')_2$ was reported to protect mice from Rift Valley fever virus infection [12].

Dixit et al. [13] concluded that equine polyclonal antibody therapies could also be adapted for widespread and severe neglected tropical diseases, for example, the viral hemorrhagic fevers like Crimean-Congo Haemorrhagic Fever, Dengue, Ebola, and Marburg; Bat-transmitted viruses such as Nipah and Hendra, as well as Lassa virus, West Nile Virus and SARS. There are studies on neutralizing antibodies against SARS-CoV-2 spike protein which has a vital role in SARS-CoV-2 infectivity [4].

A few reports related to COVID-19 animal-sourced hyperimmune globulins have been published so far [14, 15]. Rhesus macaques and golden hamsters treated with monoclonal antibodies had lower levels of detectable sub-genomic viral mRNA in both prophylactic and therapeutic experiments [2]. Four single or combination monoclonal COVID-19 antibody treatment third phase human trials have already started [16].

1 Equine and ovine sourced hyper-immune sera are already accepted and authorized as
2 routine therapies including Europe and North America [17]. Reports have been published
3 by World Health Organization (WHO) on production, quality control, and standardization
4 topics [18, 19], and regulations related to both veterinary and human use are listed in both
5 European and United States Pharmacopeia. Polyclonal antibodies have been used in
6 rabies prophylaxis, envenomation, and intoxication. They are promising treatment
7 options in emerging threats to humans [13].
8 There are some advantages of animal-sourced hyperimmune sera compared to
9 convalescent plasma. Convalescent plasma's neutralization potential needs to be tested,
10 monoclonal antibody-based immunotherapeutics are approved while they are being
11 produced [5]. Hyperimmune sera are more concentrated and include more antibodies or
12 $F(ab')_2$ [20]. Avoidance from transfusion of harmful coagulation factors and consistent
13 antibody titers are the most important advantages of hyperimmune plasma therapies over
14 convalescent plasma therapies [21].
15 Although human-derived convalescent sera may not be ready for use initially at the early
16 stages of a pandemic, animal origin hyperimmune sera can be acquired in 2-4 months in
17 large amounts with a standardized neutralizing antibody titer. Due to the production
18 similarities for all diseases, available veterinarian and human antisera producers can turn
19 out large amounts in a short time.
20 Passive antibody therapy is a prophylaxis and treatment option, moreover, horse-origin
21 immunoserum is controlled, standardized, cost-effective, and can be produced in large
22 amounts, and are logistically beneficial compared to convalescent plasma therapy for
23 low- and middle-income countries. In this paper, production and important control points
24 will be handled.

1 The aim of this study is to develop horse-specific antisera against SARS-CoV-2 for
2 emergency and low-cost response.

3 ## 2. Materials and methods

4 The Republic of Turkey Ministry of Agriculture and Forestry also started the response
5 action right after the pandemic announcement of the WHO and rapidly communicated
6 with the Ministry of Health according to one health approach and plans to develop a
7 hyperimmune serum to fight against SARS-CoV-2 were made.

8 ### 2.1. Virus isolation, production in large amounts, and inactivation:

9 SARS-CoV-2 isolates were obtained from the Health Institution of Turkey. Eight
10 nasopharyngeal swabs were also taken from Ankara 29 May State Hospital. Virus isolates
11 from the Health Institution of Turkey were used as the seed virus with alternative studies
12 carried out on three of eight swabs as a contingency plan.

13 Vero E6 cell culture maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco,
14 Grand Island, NY) supplemented with 10% foetal bovine serum (FBS, Gibco, Grand
15 Island, NY) was used in virus inoculation, and incubations were carried out in 37°C with
16 5% CO_2. First inoculations were made in 25 cm^2 cell culture flasks after adaptation of the
17 cells in 75 cm^2 and 150 cm^2 cell culture flasks. Cells were freeze-thawed after cytopathic
18 effects (CPE) were observed in each inoculation. Afterward, supernatants were collected
19 and portioned by centrifugation at 3000 rpm for 15 min. The accuracy of virus growth
20 after each inoculation was verified by Real-Time Reverse transcription polymerase chain
21 reaction (Real-Time RT-PCR). The volume of the virus was raised to 2 liters. All studies
22 using live viruses were carried out in the biosafety level 3 laboratory (BSL-3) within Etlik
23 VCCRI.

1 The QIAamp® Cador Pathogen Mini Kit (Qiagen, Germany) and Qiagen EZ1® Virus
2 Mini extraction kit v2.0 (Qiagen, Germany), and EZ1® Advanced XL workstation was
3 used with following the manufacturer's kit protocol for the extraction of viruses. By the
4 QuantiNova® Pathogen + IC Kit (Qiagen, Germany), Real-Time RT-PCR was performed
5 by using the primary probe sets [22]. Samples with amplification curves were considered
6 positive. These samples, which were detected as positive, were confirmed by performing
7 the whole-genome analysis (unpublished data).
8 Subsequently, viral suspension with a $10^{6.0\text{-}7.0}$ $TCID_{50}$/0.1 ml titer was centrifuged and
9 filtered with the intent of large-scale antigen production (Millipore, Opticap XL capsule
10 filter, 0.2 µm) and stored at -80°C until inactivation [23]. Then it was inactivated by
11 treatment with Binaryethyleneamine (Merck, Germany) + formaldehyde 37% (J.T.Baker,
12 Netherlands), only with FA and heat treatment methods [24].

13 **2.2. BEI + FA inactivation process:**

14 Before inactivation, virus suspension pH was adjusted between 7.4 ± 0.2 with 0.175 M
15 NaOH (Merck, Germany). After the temperature of the virus suspension was fixed to
16 26°C, 10% FA was added in a ratio of 1:2000 in the final product. 3% BEI solution was
17 added and continuously mixed during the process at 26°C. For inactivation follow-up,
18 samples were taken at the first five hours, $12^{th,}$ and 24th hours. Sodium thiosulfate (2%,
19 Merck, Germany) was added to all samples and final product at the end of the 24th hour
20 for terminating the FA activity. The final product and samples were stored at -80°C until
21 inactivation control.

22 **2.3. FA inactivation process:**

23 10% FA solution, adjusted to a final ratio of 1:1000 was mixed with the virus suspension
24 at a ratio of 1:1. The resulting mixture was subjected to the inactivation process for 48

1 hours at 37°C with continuous stirring. Samples were taken at 0, 4th, 8th, 12th, 18th, 24th,
2 30th, 36th, 42nd, and 48th hours for inactivation follow-up. Sodium thiosulfate (2% Merck,
3 Germany) was added to all samples taken for the termination of FA activity and to the
4 final product collected at the 48th hour. The final product and samples taken were stored
5 at -80°C for inactivation control.

6 **2.4. Inactivation by Heat Treatment:**

7 The inactivation process was carried out at 65°C. Virus suspension was placed in a 2 L
8 water bath. Virus suspension and water temperature were observed with calibrated and
9 sterile thermocouple thermometers (Fluke, Germany). The first sample was taken when
10 the suspension temperature reached 65 ± 2°C and continued every five minutes for 30
11 minutes. Later, the time was increased to one hour in total, the final product and samples
12 were taken and stored at -80°C for inactivation control.

13 **2.5. Inactivation Controls:**

14 For BEI + FA inactivation process, two flasks from each cultivation method were
15 inoculated into 25 cm^2 cell culture bottles by using adsorption and non-adsorption
16 cultivation methods from the 24th-hour sample. Each cultivation was checked for the
17 presence of CPE after a six-day incubation period and removed to -80°C. After the freeze-
18 thaw process, blind passages were carried out in three rounds by centrifugation, using
19 conventional cultivation methods. Six-well cell plates were used for the 48th hour of the
20 inactivation process with FA and the first hour of the thermal inactivation process to
21 investigate the presence of the live virus. The controls performed with the adsorption
22 cultivation method were repeated as three rounds of blind passages as described above
23 after the six-day incubation period.

24 **2.6. Immunization of the Animals:**

Totally 20 New Zealand rabbits (n: 2 for Control, 6 for BEI+FA, 6 for FA, 6 for Heat; age: 8-12 month; weight: 3-4 kg) and nine mixed bred horses (n:1 for Control, 4 for BEI+FA, 2 for FA, 2 for Heat; age: 4-9; weight: 250-350 kg) were used. All animals had a detailed clinical health assessment after quarantine and then they were included in the study. Rabbits housed in 80 cm × 60 cm × 55 cm metal cages at 22°C and had an ad-libitum feed and fresh drinking water. Horses stayed in 4.5 m × 5 m × 5 m boxes in outdoor temperature and feed with 2 kg mixed feed, 2 kg hay, and ad-libitum water without pasture.

Immunization was conducted with one injection in a week and concluded in four weeks in all animals. The first two injections with an interval of one week, 1 ml Complete Freund's adjuvant (F5881, Sigma- Alderich, Germany) and 1 ml inactive antigen was used; the next two weeks injections containing 2 ml of Incomplete Freund's adjuvant (F5506, Sigma- Alderich, Germany) and 2 ml of inactive antigen was used. Blood and plasma collections were performed at the 4th, 6th, and 8th weeks from horses to monitor the antibody response following the injections. Two rabbits were excluded from the study due to subpar health status in one and age in the other.

Microneutralization test was performed as described by [25]. 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120 and 1:10240 of serum/plasma/end product samples dilutions were subjected to neutralization at 37°C for one hour, mixing with an equal volume of SARS-CoV-2 diluted at 100 $TCID_{50}$ in duplicate for serum sample and quadruplicate for plasma samples. The tests were evaluated at the end of the fifth day under the cell culture microscope. The highest dilution step preventing the formation of CPE in 50% of the dilution chambers was accepted as the neutralizing titer. Verification was done by backward titration for each test. The test was performed by using two wells

per serum sample and four wells per plasma sample. Results were converted to Log_{10} and displayed graphically. Rabbit products were not used for further steps.

Plasma samples were collected from horses, whose titers were found suitable at the end of antibody titer controls after immunization, in a completely closed system with the plasmapheresis device. Collected plasma (totally 75 L) pooled and tested for extra zoonotic agents according to the WHO guideline [18]. After then, to collect $F(ab')_2$ fragments from pooled sera, the plasma purification process was carried out according to WHO's protocol with 2% pepsin and 2-5% caprylic acid [18].

2.7. Final product control:

All tests were conducted according to Immunsera for human use chapter of European Pharmacopeia 10.0 and related methods. Impurity controls were done by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method in commercial laboratories (DFA and Sabancı University) according to Ng et al. [24]. Samples were loaded from the stock into the wells at dilution of 1:1, 1:5, and 1:10 as shown in Figure 1. In SDS-PAGE analysis, it was aimed to determine the impurities more clearly by loading a higher amount of sample. SDS-PAGE analysis carried out under reducing and non-reducing conditions according to Ng et al. [24] with protein standard ladder (Precision Plus Protein™ WesternC™ Blotting Standards, BioRad, Germany) and according to the kit protocol (FastCast™ Acrylamide Kit, BioRad, Germany) as shown in Figures 1-2.

To the samples prepared under non-reducing conditions, 2-mercaptoethanol was not added and the samples were not boiled. For reducing conditions, 2-mercaptoethanol was added to the samples and they were boiled. The 90 kD band of 1:50 dilution in non-reducing gel and 23 kD band of 1:50 dilution in reducing gel were taken as reference and

corrections were made according to the dilutions while evaluating the bands in different wells together. It was observed that the amounts of protein bands in the different wells were consistent after dilution correction.

In vivo pyrogenicity test was carried out in 1.5 kg, adult, Albino rabbits according to European Pharmacopeia 10.0 chapter 2.6.8, ISO 10993-2:2006 Biological evaluation of Medical Devices: Animal Welfare Requirements and ISO 10993-12 2012 Biological Evaluation of Medical Devices Sample Preparation Standards by Scientific-Technological Application and Research Centre of Kırıkkale University. This test is based on the evaluation of the increase in rectal body temperature after intravenous administration in rabbits, depending on the bacterial endotoxin content of the final product.

To assess the safety of the product, 1 ml of the final product was intraperitoneally injected into 22-25 g Swiss albino mice. A total of three batches, including the control group, were made using both pure (undiluted) form and 1:3 diluted form as the final product. Mice had *ad libutum* feed and water. They were weighed on the first, third, fifth, and seventh days.

The final product was controlled in terms of zoonotic and horse-borne diseases in Etlik VCCRI according to the WHO guideline [18]. The protein level of the final product was calculated by the Doumas method by Leco® FP 521 (Leco, USA) in Ankara Food Control Laboratory [26]. Protein content was calculated by multiplying the resulting nitrogen content by 6.25.

2.8. Statistical analysis:

Grubb's test was performed to identify outlier results at the beginning of safety tests. After controlling individual weights that showed a normal distribution, Levene's test was used

to check the homogeneity of variances. The results were evaluated by the General Linear Model with repeated measures method in terms of time and group difference. The resulting differences were evaluated against the control group in pure and 1:3 diluted groups by two-way Dunnett's test. The limit of statistical significance was assumed as $p < 0.05$. Statistical analyses were conducted with IBM® SPSS® (V.21).

3. Results

The virus strain (GenBank: MW306666) used in this study was taken from the Health Institution of Turkey and was produced and subsequently inactivated in large volumes in the Etlik VCCRI BSL-3 laboratory.

In the inactivation controls, it was determined that the inactivation was completed within the specified time in both BEI + FA and FA inactivations. In the 30th minute of the thermal inactivation process, it was determined that the process was insufficient since CPE development was detected, and therefore, an additional 30 minutes of heat treatment was applied on cell culture. Following this one-hour inactivation process, there was no CPE development on cell cultures.

No clinical health problems or adverse effects were observed throughout the study in horses and rabbits. Horse Body Condition Score remained constant throughout the study (between 5-6/9). No feed additive used in this study and the crude protein content of the rations were 7.1% and 18.4% respectively, in horses and rabbits.

Serum samples taken from all horses and rabbits at the beginning of the immunization were checked for the presence of neutralizing SARS-CoV-2 antibodies and all were found negative. Neutralizing antibody formation kinetics were observed following immunization. Antibody responses began to be detected in horses and rabbits from day 21. Following the third immunization (28th day), there was 14-fold in antibody titers of

horses as shown in Figure 3. Two of the horses had the highest titer four weeks after the first immunization, while the other four horses had the highest titer in the 6th week following immunization. It was found that high neutralizing antibody titer values were maintained in six out of eight horses in the eighth week. There was no difference between BEI + FA and FA inactivation methods in terms of the rate of increase in antibody levels, but higher antibody levels were achieved in horses immunized with BEI + FA as shown in Figure 3.

As a result of the inactivation and immunization studies carried out by our institute, the titer values obtained from horses varied between 1:640 (= 2.8 $\log_{10}$) and 1:5120 (= 3.7 $\log_{10}$) at the individual level, and the neutralizing antibody titer value of the product obtained after purification was determined as 1:1280 (= 3.1 $\log_{10}$).

When the rabbits were evaluated in terms of these differences, it was determined that they reached the highest titer values in the sixth week following the fourth immunization and higher titer values occurred in rabbits immunized with the antigen inactivated with BEI + FA in accordance with the values in horses as shown in Figure 4. Antibody response was not obtained in horses and rabbits immunized by the thermal inactivation method. In rabbits and horses immunized with BEI + FA and FA inactivated antigen, mean titer values of horses were found to be higher than rabbits as shown in Figure 5. Neutralizing titer amount of BEI +FA group was higher than FA group ($p = 0.05$). There was no statistically significant difference in time-dependent variation of titers occurring in rabbits and horses ($p = 0.09$). When the titer changes were examined based on time, the titer values on the 21st day were found to be statistically different from the other days ($p < 0.05$), but no significant change was observed between the following days.

The titer value obtained at the end of the virus neutralization test performed after the concentration and purification process of the plasma collected as 75 L was determined as 1:1280. It was determined that the final product obtained in the tests of sterility and extra agent controls was free from bacterial and extra agents.

It has been determined that the dominant component in the antiserum content is $F(ab')_2$ derived from an immunoglobulin. Direct mass analysis was performed by MALDI-TOF for the detection of different proteins in the final product as shown in Figure 6. The peaks seen in the analysis show the protein weights of the components. Other peaks were judged to be contaminating proteins from horse serum. As a result of the analysis, it was determined that the dominant component in the antiserum sample was $F(ab')_2$ originating from an immunoglobulin. Proteomic analysis and SDS-PAGE analysis support this finding.

When the results of gel analysis and molecular sieve chromatography performed with the SARS-CoV-2 antiserum under reducing and non-reducing conditions are interpreted together with the results of the mass analysis; It has been determined that 84-86% of the total protein in the antiserum consists of various immunoglobulins in the range of 90-200 kD and 14-16% of the proteins in the antiserum are in the range of 10-50 kD. The detected percentages were consistent with each other in the gel electrophoresis analysis, molecular sieve chromatography results, and electrophoresis results with the separated proteins made from the original undissociated antiserum. It is thought that the band seen around 47 kD in the 1:10 dilution well containing a high amount of protein in non-reducing gels may be the IgG heavy chain. In the analysis made with separated proteins, the total amount of protein bands between 37-50 kD was determined as 6% and the number of

proteins between 10-20 kD was determined as approximately 6%. It has been found that protein bands between 10-50 kD can be identified by suitable methods, eg, mass spectroscopy or affinity chromatography. Osmolarity value was determined as 324 mOsm/Kg H_2O and 13% nitrogen was calculated by the Doumas method [26].

The first day of the safety test was recorded as Day 0. The mice were then weighed on the first, third, fifth, and seventh days and weight gains were evaluated relative to the control group. The weight changes in the pure (undiluted) and 1:3 diluted final products were not found to be statistically significant. It was observed that the weight gain of the experimental groups were not different from the control group within the week. *In vivo* pyrogenicity test results concluded that the pure undiluted antiserum sample did not have any pyrogenic effect.

4. Discussion

The emergence of the SARS-CoV-2 has brought many challenges in terms of health. There is currently no vaccine or drug therapy known to be effective against this virus. The disease is kept under control with measures such as early and rapid diagnosis, quarantine, and follow-up. This epidemic that started around the world created heavy treatment costs. For these reasons, a need has arisen for easily accessible and low-cost treatment options in all healthcare fields. In addition to convalescent plasma therapy, the importance of which has increased in recent years, antisera, which have a long history and are actively used in the treatment of rabies infection, is being considered an alternative treatment method in SARS-CoV-2.

Horses and rabbits were cared for throughout the entire study, kept free from stress, and with attention to their well-being. In the ration of a non-working horse weighing 250 kg, literature recommend at least 270 g of crude protein per day [27, 28]. In rabbits, the

recommended amount of 14-17% crude protein is slightly exceeded [29]. The amount of antibody increased with the amount of crude protein used in 142 g/day horses and 18% rabbits. It is thought that reporting the characteristics of the rations used in such studies in more detail will contribute to future studies.

Among the three different inactivation methods used in the study, the study in which the highest immune response was obtained in both animal groups was the method performed with BEI + FA. There are very few publications that have performed SARS-CoV-2 inactivation and animal immunization using both inactivates. For this reason, a detailed comparison based on other sources could not be made. However, considering that there may be individual differences between animals, this result should be evaluated in detail with a more controlled observation process.

In the study conducted by Zylberman et al. [14] in Argentina, the receptor-binding region of the spike protein belonging to the SARS-CoV-2 was amplified by recombinant applications and the proteins obtained from the cells were purified by the affinity chromatography method and used in horse immunizations. The results obtained indicated that an antibody titer of 1:10240 was reached in horses and that this titer was highly preserved at the end of the subsequent plasma purification processes, so the final product contained neutralizing antibodies at a titer approximately 50 times higher than the convalescent plasma. Lu et al. [10], in a study using live SARS-CoV, reported that up to 1:14210 serum antibody titer was reached in the 7th week following the first immunization in horses. Following purification, this titer was determined to be 1:5120.

As a result of the immunization studies performed with the inactive SARS-CoV-2 carried out by our institute, the titer values obtained from horses varied between 1:640 - 1:5120 at the individual level, and the neutralizing antibody titer value of the product obtained

after purification was determined as 1:1280. Convalescent plasma titer level is found to be acceptable at 1:160 by The Food and Agriculture Organization (FAO), and 1:80 when it is not available.

SARS-CoV-2 inactivated by gamma rays was used to inoculate horses (n: 10, Age: 4-10 years) and highly neutralizing antibody titers were detected by ELISA and the purity was checked by SDS-PAGE [30]. The purity of the antisera was determined to be 99% with a neutralizing titer of over 20000 [30]. This study also supports our findings and promises effective results for neutralizing the SARS-CoV-2 *in vivo*.

In order to investigate the effects of receptor binding domain fragments made by plasmids, after three immunizations performed with Freud's and incomplete Freud's adjuvant within two weeks, the product was purified and it was determined to be 20-100 times more potent like in our study [31].

In another study, horses were injected with trimetric spike glycoprotein, a method used in anti-rabbit antisera, and antibody response was approximately 150 times higher than antibody convalescent serum in horses [32]. This method provided higher immunogenic potency than our method.

León et al., [15] injected two groups of three horses each, with either S1 (anti-S1) or a mixture of S1, N, and SEM mosaic (anti-Mix) viral recombinant protein, and the maximum anti-SARS-CoV-2 polyclonal antibody level (approximately 80 times more than convalescent serum) was reached at the end of seven weeks. The results obtained indicated that an antibody titer of 1:25355 was obtained for anti-Mix in horses.

A similar study against Middle East Respiratory Sendrom was conducted by Gai et al. [33]. In this study, again VLP was used, but rodents were used to obtain hyperimmune serum in the study. The results obtained by these researchers gave similar results to

Schimt et al. [34]. However, at the end of the challenge tests performed in mice and guinea pigs, it was emphasized that Fc-dependent antibody mechanisms could play an important role in this process in addition to virus neutralization, where the timing of therapeutic administration is critical.

In order for these antibodies to be used primarily in human health, it is necessary to purify the antiserum by removing foreign proteins of horse origin that cause side effects that can lead to anaphylactic shock. For this, it is recommended to test with immune-affinity columns in future studies, but it is thought that costs will also play an important role in decision-making in these applications.

Since the rabbit model used before horses gives similar results in our study, it may be very advantageous to work with rabbit models before horse studies to prevent waste of time, labor force, and resources.

Monoclonal antibody production is expensive and requires know-how and equipment. This technique is used for different diseases so far and is still known as one of the safer and cost-benefit methods in this field. This process will be one of the efficient, precise, and safe methods against COVID-19.

There are some limitations in our study. First of all, in an environment where the borders were closed and access to raw materials were very difficult, our studies after blood collection from horses were carried out in a company with veterinary Good Manufacturing Practices. This situation has raised the question of whether a product produced in veterinary facilities can be used for human health. Our study has shown that in such emergencies, veterinary production sites are indeed suitable for human health products. As in the case of current pandemic, organizations such as the WHO, FAO, and/or World Organisation for Animal Health by making emergency action plans together

1 with their sub-institutes, documenting and updating them regularly, can eliminate the
2 confusion of authorization in future emergencies, thus bringing the world one step closer
3 to gathering under a single health umbrella. The most important output of this study in
4 times of such a crisis is that this project, which was organized in a short time with the
5 contribution of many institutions related to their subjects, has been completed with one
6 health approach.

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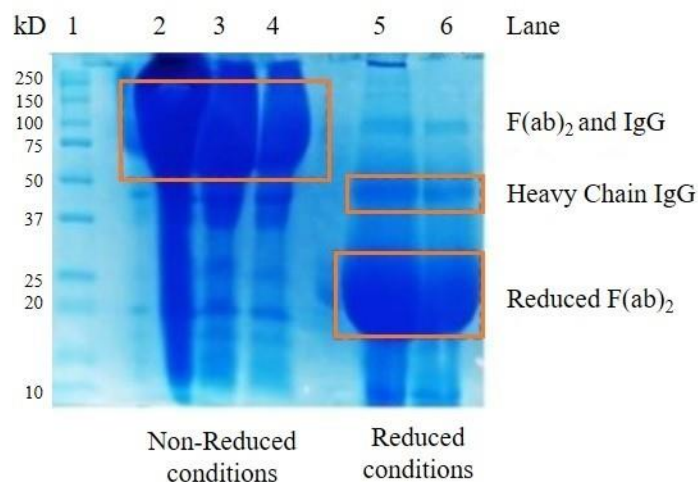
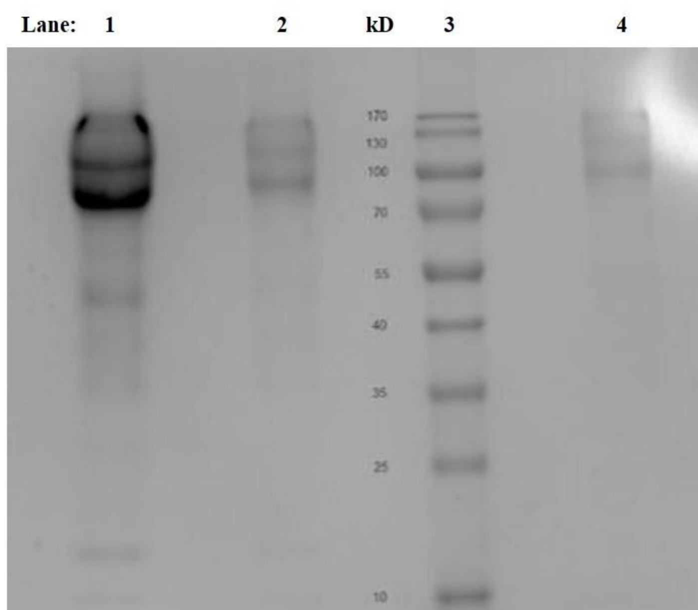
4 **Figures**

5



6

7 **Figure 1.** Lane 1: Protein standard Ladder (Molecular weight, kD). SDS-PAGE
8 analyses were carried out under non-reducing (Lane 2: 1:1, Lane 3: 1:5, Lane 4: 1:10)
9 and reducing (Lane 5: 1:5, Lane 6 1:10) conditions.



10

1 **Figure 2.** Results of 8-16% SDS-PAGE analysis from final SARS-CoV-2 antiserum

2 product prepared under non-reducing conditions (Lane 1: 1:10; Lane 2: 1:50; Lane 3:

3 Protein standard Ladder; Lane 4: 1:100).

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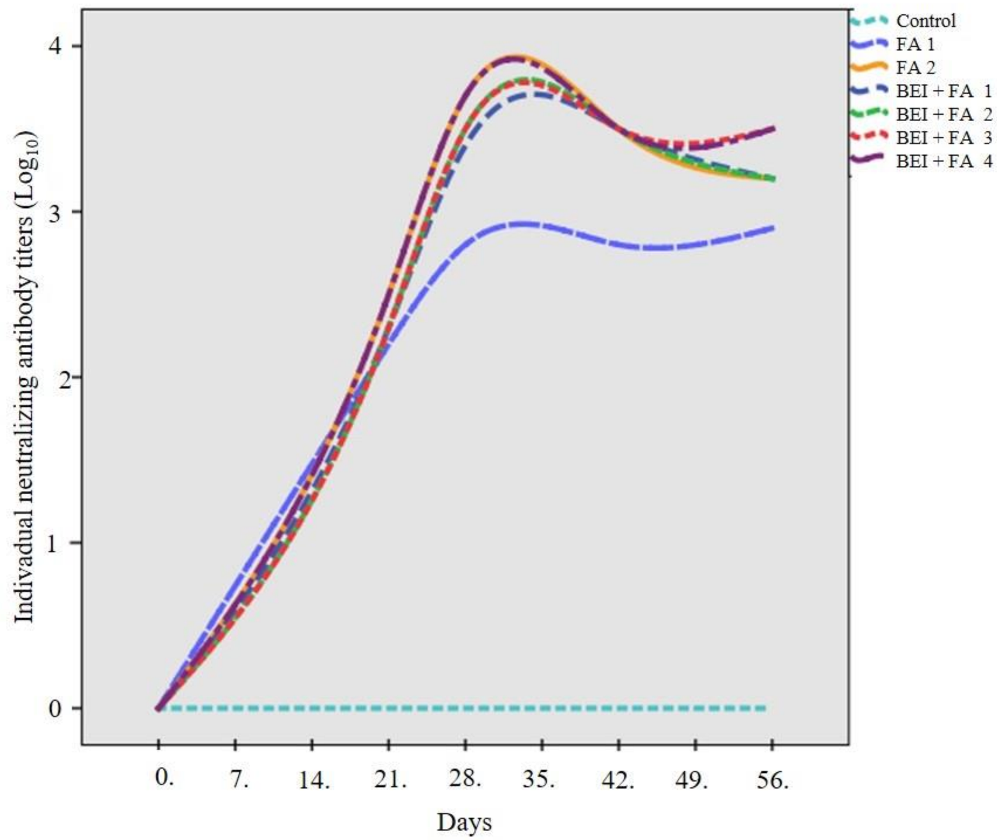

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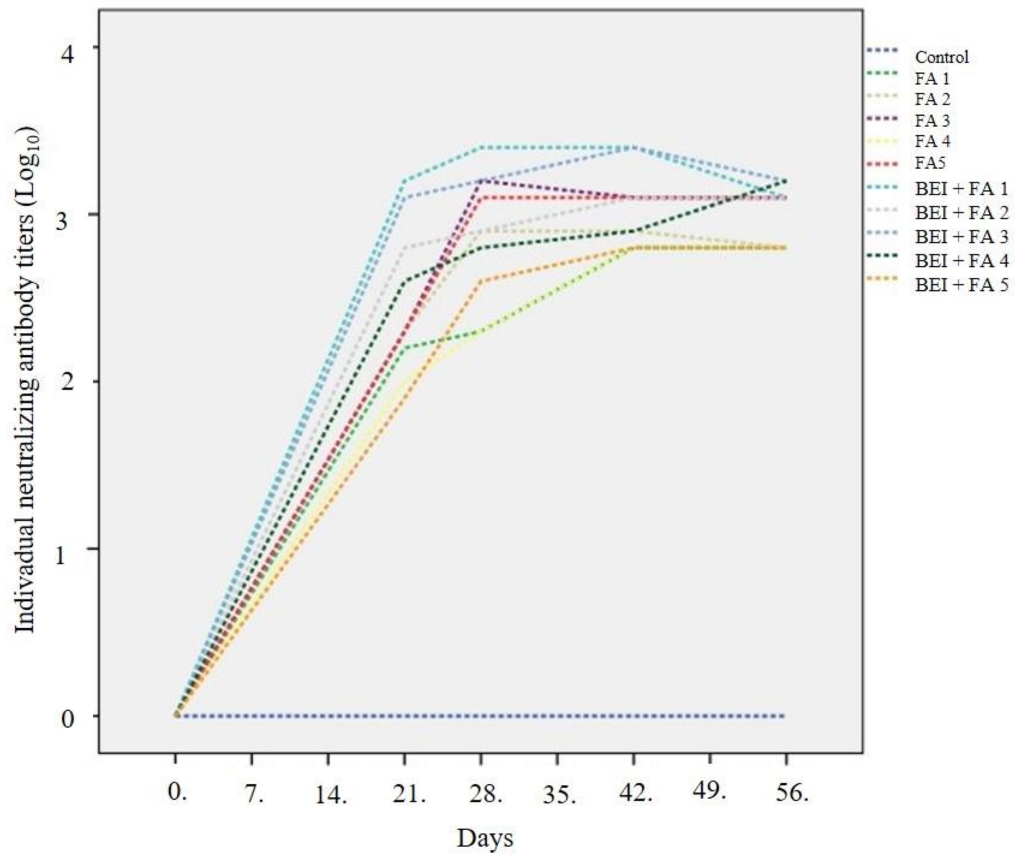
6 **Figure 3.** Individual neutralizing antibody titers (Log_{10}) of horses in FA and BEI + FA

7 groups in 56 days.



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2 **Figure 4.** Individual neutralizing antibody titters (Log_{10}) of rabbits in FA and BEI + FA

3 groups in 56 days.

4

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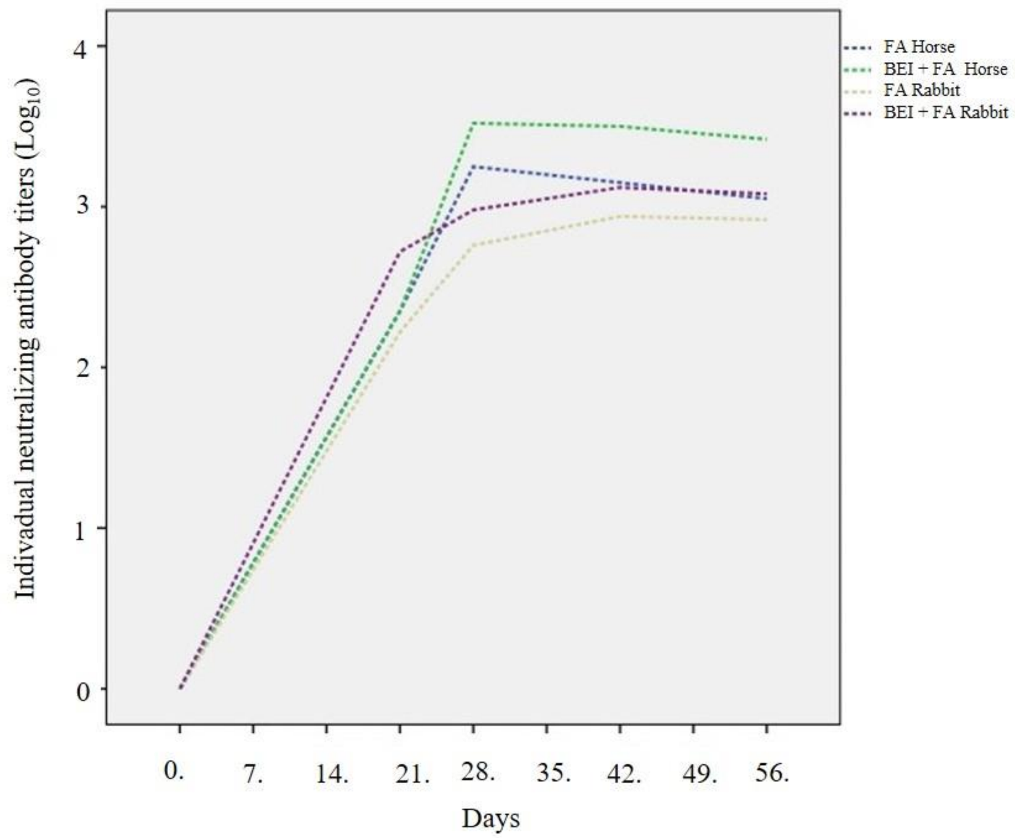

2 **Figure 5.** Horse and rabbit mean neutralizing antibody titers (Log_{10}) of FA and BEI +

3 FA groups in 56 days.

4

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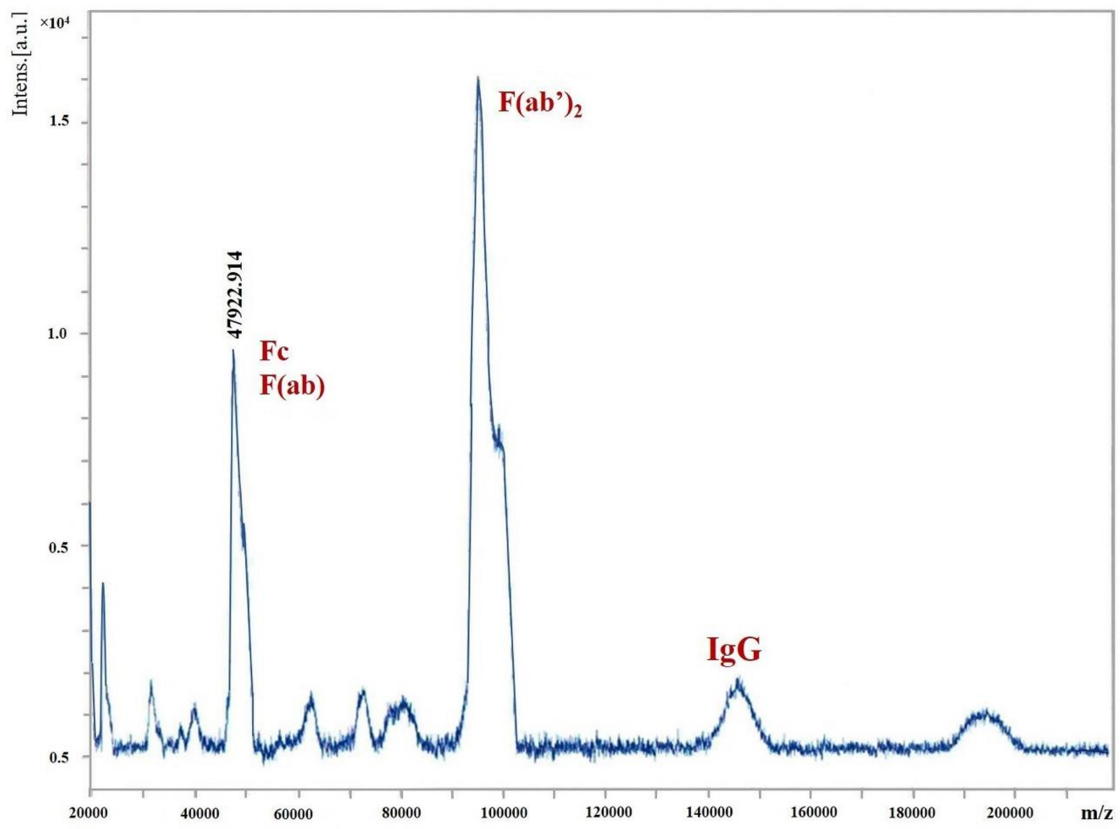

2 **Figure 6.** MALDI-TOF results showed its components with the highest level of F(ab')$_2$.

3