Development of equine antisera with high neutralizing activity against SARS-CoV-2

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Research Article

Keywords: SARS-CoV-2, equine hyper immune globulin, F(ab')₂ fragments, neutralizing antibody

Posted Date: September 29th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-83582/v1>

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Abstract

The pandemic of COVID -19 caused by SARS-CoV-2 is leading to a humongous impact on the mankind with over a million people succumbing to it worldwide. Although there are few drugs approved for the treatment, there is not yet a safe and effective vaccine available for COVID-19. Also, the passive immunization therapy with convalescent plasma, though potentially an effective treatment option for other viral disease has limitation of availability. The prior use of immunoglobulins generated in animals has proven to be effective in several viral and bacterial diseases. Here, we report the development and evaluation of equine hyper immune globulin raised against inactivated SARS-CoV-2 virus. Post immunization neutralization titres of the equines demonstrated high neutralizing antibodies. To minimize the adverse effects, the immunoglobulins were digested with pepsin, and purified to obtain the F(ab')₂ fragments. The average nAb titre of the purified bulk was 22,927 and correlated with high IgG binding efficiency in ELISA. The quality control assessments of the different batches proved to have consistent nAb titres. The study provides evidence of the potential of generating highly purified F(ab')₂ from equines against SARS-CoV-2 that can demonstrate consistent and high neutralization activity. Further, *in-vivo* testing for efficacy of this indigenously developed, cost effective product will pave the way to clinical evaluation.

Introduction

Recently, a novel coronavirus was documented during an outbreak of viral pneumonia among human population of Wuhan city, Hubei Province, China in December 2019¹. A high rate of human-to-human transmission was observed that lead to the spread of the virus within Wuhan, China and other countries. Considering the severity of the infection, and the rapid spread to several countries, World Health Organization (WHO) declared the outbreak of 2019 novel coronavirus (2019-nCoV) a Public Health Emergency of International Concern (PHEIC) on 30 January 2020 and further recognized it as a pandemic on 11 March 2020. Until now, a large number of people are affected with SARS-CoV-2 and approximately a million have succumbed to the disease across the globe².

Although there are few drugs approved for the treatment and many under different phases of trial^{3,4}, there is not yet a safe and effective vaccine available for COVID-19. Prophylactic and /or therapeutic intervention strategies would be the most appropriate and effective method for control of the rapid spread of this dreadful disease. Immunoglobulins are well-known for its therapeutic property against many diseases. These immunoglobulins are produced using the inactivated pathogen or toxins as an immunogen to immunize equines to generate hyper-immune serum containing polyclonal IgG's. The practice of administering purified polyclonal immunoglobulins (IgG) from hyperimmune sera of animals has been used extensively in the successful control of many viral and bacterial infections i.e. Rabies, Cytomegalovirus, Hepatitis B, Vaccinia virus, Tetanus, Botulism, Diphtheria etc.^{5,6,7}. A few studies have reported the equine hyperimmune sera against SARS-CoV-2 S1 RBD proteins as being potent because of generation of high titers of neutralizing antibodies^{8,9}.

Passive immunization of severely ill COVID-19 patients with plasma from recovered COVID-19 patients was reported to be an effective treatment option^{10,11}. The US Food and Drug Administration (FDA) issued an emergency use authorization (EUA) of convalescent plasma for the treatment of hospitalized COVID-19 cases on August 23, 2020¹². However, obtaining the plasma from recovered patients is a difficult task and its titer and quality keep changing from one patient to other. In such situations, production of antiserum in large animals is a good alternative. Considering the need for effective therapeutics, we have developed and evaluated a SARS-CoV-2 (COVID-19) antiserum immunoglobulin (Purified F(ab')₂ fragments) against SARS-CoV-2.

Materials And Methods

Ethical statement:

The study was approved by the Institutional Animal Ethics Committee and further approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi letter number V-11011(13)/7/2020-CPCSEA-DADF.

Virus strains

Virus propagation and titration:

SARS-CoV-2 (NIV-2020-770) strain was isolated from throat/nasal swab specimen of COVID-19 positive patient in Vero CCL-81 cells at the maximum containment facility of ICMR-NIV, Pune¹³.

SARS-CoV-2 stock was prepared by inoculating the known titer of virus in three passages in Vero CCL-81 cells. Cytopathic effect was first observed on second post-infection day (PID) and harvested on third PID. Virus titrations were performed in Vero CCL-81 cells using tissue culture infectious dose 50% (TCID₅₀) assay. Virus titre (TCID₅₀/ml) was calculated by the Reed-Muench method and found to be 10^{6.5} TCID₅₀/ml.

Antigen preparation

Gamma inactivation of the virus: Gamma irradiation of the virus stock was performed using Co-60 source (24 kGy) of GC-5000 Gamma chamber (BRIT, Mumbai). This irradiated stock was again inoculated in Vero CCL-81 twice and observed for five days to confirm the complete inactivation of the virus (Elliot et al., 1982)¹⁴.

Concentration of gamma-inactivated antigen: Gamma irradiated SARS-CoV-2-infected tissue culture fluid was concentrated using 30 kDa filters (Pall, Germany) and further passed through 0.2 µm filters, aliquoted and stored at -80°C. Concentrated viral antigen was also aliquoted in 1 and 2 ml volumes in

frosted glass bottles and further lyophilized. The lyophilized vials were stored at -20°C to be used as a source of whole virus antigen

Equine Immunization:

Ten 4–10 years old, healthy equines (160–200 kg in weight) that had no detectable antibodies against SARS-CoV-2, were chosen for primary immunization at Biologicals E. Ltd (Bio E). The equines were numbered HK1 to HK10, and the same numbers were used to represent the plasma obtained from individual equines accordingly. Equines were inoculated with inactivated SARS-CoV-2/VeroCCL81/P-4 antigen subcutaneously along with Freund's adjuvant. After completion of initial immunization, the equines were test bled and plasma samples were collected and anti-SARS-CoV-2 IgG was tested via ELISA and plaque reduction neutralization assay (PRNT).

Enzyme-linked immunosorbent assay (ELISA)

Specificity of antibodies raised in equines were evaluated by sandwich ELISA using inactivated SARS-CoV-2 antigen (Sapkal *et al.*, 2020)¹⁵. Briefly, 96-well polystyrene microtitre ELISA plates (Nunc, Thermo Fisher Scientific, USA) were coated with inactivated SARS-CoV-2 antigen (1:10 diluted, 100 μl /well) in 1x Phosphate-Buffered Saline (PBS) (pH 7.2), overnight at 4°C and then were blocked with a 1% BSA in 1x PBS for one hour at 37°C . The plates were washed three times with 1x PBST, pH 7.4 with 0.1 % tween-20 (PBST). To the coated plate, 100 μl of 1:100 diluted equine plasma samples were added and incubated at 37°C for one hour. After each step, the plate was washed five times using 1x PBST. Following this, 100 μl /well of anti-horse IgG horseradish peroxidase (HRP, Sigma-Aldrich, USA) (1:16000) diluted in 1x PBST and added; and plates were incubated for one hour at 37°C . Further, 100 μl of 3, 3', 5, 5'-tetramethylbenzidine (TMB, Cellbiosis) substrate was added and incubated for 10 min. The reaction was stopped by adding 100ul of 1N sulphuric acid (H_2SO_4), and the absorbance values were measured at 450 nm using an ELISA reader (Thermo Fischer scientific, USA). Normal horse plasma (non-immunized) was used as negative control and pooled plasma of 10 immunized animals been used as positive control. The cut-off for the assay was defined as mean of negative control optical density plus three standard deviation (3SD).

Further to identify the binding efficacy of the antibodies, multiple dilutions of pooled plasma collected from the equines and the purified bulk preparations produced from the plasma were tested in ELISA. For negative control, normal horse serum (non-immunized) was added. The bound antibodies were then probed with anti-horse IgG-HRP conjugate. The end point titre was defined as the reciprocal of the highest dilution of the sample that gives result above the cutoff¹⁶.

Plaque reduction neutralization test (PRNT): PRNT was performed as described by Deshpande *et al.*, (2020)¹⁷. Briefly, four-fold serial dilutions of heat inactivated (56°C for 1 h) horse plasma samples were mixed with an equal amount of virus suspension containing 50-60 plaque forming units (pfu) in 0.1 ml. After incubating the mixtures at 37°C for one hour, each virus-diluted plasma sample (0.1 ml) was inoculated onto one well of a 24-well tissue culture plate containing a confluent monolayer of Vero CCL-

81 cells. After incubating the plate at 37°C for one hour, overlay medium (2% CMC with 2% FBS in 2× MEM) was added to the cell monolayer, and the plate was further incubated at 37°C in 5% CO₂ for 4 days. Plaques were observed and the plates were stained with 1% amido black for an hour. Antibody titres were defined as the highest plasma dilution that resulted in >90 per cent (PRNT₉₀) reduction in the number of plaques.

Purification of SARS-CoV-2 F(ab')₂ equine immunoglobulin

Plasma collected from the 10 equines was pooled to produce a plasma pool and was used to produce multiple batches of purified F(ab')₂ fragments as per established manufacturing technology at BioE at pilot scale. In the first step, the plasma pool was diluted and the pH adjusted to 3-3.5 and enzyme pepsin was added to initiate IgG digestion. During the process, the pH and temperature was controlled to ensure complete digestion. After the completion of the enzymatic reaction, the pH of the solution was raised and heated till 55°C to inactivate residual pepsin as well as any equine viruses potentially present in the plasma. After heat inactivation, caprylic acids were added to precipitate contaminating plasma proteins such as albumin. The precipitated proteins were removed from the solution via cloth filtration. The filtered solution containing F(ab')₂ fragments was then further purified using ultrafiltration-diafiltration and formulated into a glycine-sodium chloride buffer. The formulated bulk was filtered through a 0.2 micron filter to produce Purified Bulk F(ab')₂ fragment immunoglobulins.

Two to three batches of purified bulk were pooled and mixed with the glycine-sodium chloride buffer and then sterile filtered to produce final bulks. The final bulk preparations were again sterile filtered in an on-line manner on an automated vial filling line and filled into 2R glass vials stopper with rubber bungs and sealed with flip-off aluminium caps to produce final lot product suitable for clinical evaluation. Non-reducing SDS-PAGE gels, using the buffer system described by Laemmli (1970)¹⁸, were used to monitor the digestion process.

Overall scheme of immunization strategy and equine hyperimmune globulin production has been represented in **Figure-1**.

Quality control assessment of the purified equine anti-SARS-CoV-2 Immunoglobulin.

Physical and biochemical properties of equine anti SARS-CoV-2 final bulk and final lot Immunoglobulin was determined as per the standard guidelines and in-house specifications for clinical product characterization^{19,20,21,22} (**Supplementary data**).

Data Analysis

The data analysis were performed with IBM SPSS statistics 20 (NY, USA) and GraphPad Prism 8 (San Diego, CA, USA). The neutralizing endpoint as the reduction in the number of plaque count by 90 per cent (PRNT₉₀) was calculated by probit analysis. ELISA results (OD) were plotted as mean OD for each sample.

Results

SARS-CoV-2 specific IgG and neutralizing antibody response

Ten healthy horses were immunized with inactivated SARS-CoV-2 virus subcutaneously and after 21 days of immunization, plasma samples were tested by anti SARS-CoV-2 IgG ELISA and PRNT for the detection of nAb titres. The results of the plasma samples obtained from HK1 to HK10 indicated the presence of SARS-CoV-2 specific IgG antibodies as detected in ELISA with neutralizing capacity. (Figure 2).

Further, the neutralization activity of the plasma pooled from the ten equines after four rounds of immunization were tested for the presence of nAb by PRNT. The nAb titre of the pooled plasma was >4096 (last dilution of antibody tested). From the pooled plasma, seven lots of purified bulks were prepared and the purified bulk batches were tested for anti-SARS-CoV-2 binding IgG antibody by ELISA and nAb by PRNT to recognize the variation of antibody titres between the batches, the results are represented in **Figure-3**.

Purification and characterization of F(ab')₂ from pooled plasma:

Two or three of the purified bulk preparations were pooled and then sterile filtered to generate three final bulk preparations. Each final bulk was further sterile filtered and filled into 2R glass vials on an automated filling line. During the filling process, vials were collected at the beginning, middle and end stages. Each final bulk and the filled vials produced from the final bulk were tested for PRNT₉₀ titers as summarized in Figure 4.

The nAb titers are consistent from Final Bulk to Final Lot (filled vials) across entire filling operation during all three batches.

Preparation of F(ab')₂ : Purity of F(ab')₂ of Final bulk and Final lot samples was assessed by SDS-PAGE to check key plasma impurities such as albumin. The SDS-PAGE profile shows the F(ab')₂ fragment band at molecular weight of approximately 100 kDa. Albumin was added in multiple lanes at 0.5 to 8 mcg quantity corresponding to 0.5% to 8% of relative impurity content in the final bulk and final lot samples (**Figure 5**).

Measurement of IgG titres in purified plasma by Anti-SARS Co-V2 ELISA

The results of ELISA indicated the good specificity of the antibodies raised in equines against SARS-CoV-2. The binding efficacy of the antibodies was also evaluated by this ELISA which showed a capacity of recognizing antigen at the highest dilution of 1:81920 of equine sera (**Figure 6**).

Quality control assessment of the purified products

The quality of three batches of the final bulk and final lot were checked as per the standard criteria. The samples were checked for appearance, pH, total protein content, osmolality, purity, molecular size distribution, albumin percentage and a few others. The pH of the final bulk and lot for all the three batches were between 6.37 to 6.49 for bulk and 6.45 to 6.55 for the final lot. The overall purity of the F(ab')₂ fragments produced from plasma processing was evaluated by SDS-PAGE and SEC-HPLC (data not shown) and all the three batches of final bulk and the final lots produced from these bulks showed consistent purity of 99%+ and minimal content of impurities such as aggregates and other plasma proteins such as albumin. The detailed descriptions of the quality check criteria are in the appendix **(Supplementary tables 1 and 2)**. Pooled plasma and the three final lot batches produced from the plasma were tested by a validated PCR method for a panel of equine viruses and all were absent indicating appropriate inactivation during the process (data not shown).

Discussion

In the current state of the pandemic, due to the unavailability of approved specific vaccines and drugs for treatment of SARS-CoV-2, an urgent need of therapeutic strategies are required. Human convalescent plasma has not met with the desired results⁸. Purified immunoglobulins obtained from hyper-immune equine sera has been an effective and time- tested approach in various infections such as diphtheria, tetanus, rabies and bites from snakes, scorpions, arachnids and more recently SARS-CoV-1, MERS-CoV, Ebola and avian influenza virus^{23,24,25,26}.

The enzyme purified equine F(ab')₂ without FC region further reduces side effects and makes it more suitable for use as a therapeutic agent to neutralize the pathogen. However, being a heterologous protein it may be susceptible to immune resistance by the host; hence a large therapeutic dose may be required for human administration. Further, the equine antibodies acts as broad-spectrum antiviral drugs due to its property of multi target action²⁷.

In this study, we report the preparation of equine hyper-immune sera to demonstrate their protective efficacy against SARS-CoV-2 virus using an *in vitro* live virus neutralization assay.

The antiserum was prepared by injecting inactivated whole virus antigen in horses subcutaneously for a period of 21 days. The resulting nAb titres in the plasma of the immunized equines displayed high titres against SARS-CoV-2. Since heterologous antisera can cause adverse effects as reported earlier in case of SARS-CoV-1, these antibodies were fragmented by pepsin to purify the F(ab')₂ fragments. F(ab')₂ from three batches of hyper-immune sera generated from ten equines showed similar protective effects against live virus. As was evident during the study, the neutralizing activity of the antibodies was preserved during the enzymatic digestion process that yielded highly purified and effective F(ab')₂ fragments. The purity of the antisera was recorded as 99 % and the neutralizing titres above 20,000.

Our results are in agreement with the other studies on equine antisera for SARS-CoV-2 which reported generation of high nAb in horses against receptor binding domain of the spike protein of the virus. This

study suggests promising efficacy and therapeutic potential of equine hyper immune sera against SARS-CoV-2. Equine hyper-immune serum overcomes the challenge of limited availability of convalescent plasma from recovered patients. Monoclonal antibodies on the other hand are laborious and expensive to generate. Equine antiserum is now known to be safer, since it is devoid of the Fc domain of antibody and can be prepared in bulk at a lower cost.

Conclusion

The study provides evidence of the potential of generating highly purified F(ab')₂ from equines against SARS-CoV-2 that can demonstrate consistent and high neutralization activity. Further, *in-vivo* testing for efficacy of this indigenously developed, cost effective product will pave the way to clinical evaluation. Additionally, being a donor independent method, this may prove as an efficient alternative to convalescent plasma for treatment of COVID19 patients.

Declarations

Acknowledgment: Authors acknowledge the encouragement and support extended by Prof. (Dr) Balram Bhargava, Secretary to the Government of India, Department of Health Research, Ministry of Health and Family Welfare, and Director-General, Indian Council of Medical Research (ICMR), New Delhi. Authors also acknowledge Ms. Mahima Datla, Managing Director, Biological E. Limited, Hyderabad for her support. Authors thank the staff of ICMR-NIV Pune for their excellent technical support including Dr Sharda Sharma, Mr. Prudhvi Lal Bhukya, Mr. Chetan Patil, Mr. Prasad Gomade of Diagnostic Virology Group, staffs of Maximum Containment facility Mr. Rajen Lakra, Mr. Prasad Sakale, Mr. Deepak Mali. Ms. Savita Patil, Ganesh Chopade, Manjunath Holleppanavar, Ms Kaumudi Kalele, Hitesh Dighe, Mr. Sanjay Thorat, Mr. Ratan More, Mr. Madhav Acharya, Ms Malvika Salave, Mrs.Ashwini Baghmare, Ms Ciyona and Ms Deepika Chowdhary, Director Office, for her administrative support.

Conflicts of Interest: None.

Financial support & sponsorship: Intramural funding support by Indian Council of Medical Research, New Delhi for COVID19 and Biological E. Limited, Hyderabad

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Figures

Figure 1

Scheme of immunization strategy and equine hyperimmune globulin production

Figure 2

Anti-SARS-Co-V2 IgG antibody detected by enzyme linked immunosorbent assay and neutralizing antibody (nAb) activity in 10 equines immunized with SARS-CoV-2 inactivated virus antigen: Horse-K1 to horse-K10 (Plasma collected after three rounds of immunization)

Figure 3

Anti-SARS-Co-V2 IgG antibody detected by enzyme linked immunosorbent assay and neutralization test of purified bulk equine plasma of seven batches (Average neutralizing antibody titer-PRNT90 is: 22,927)

Figure 4

nAb titers of final bulk preparations and corresponding Final Lot samples collected during the filling process (Initial, Middle and Final stage of the filling process) demonstrating consistency of the overall vial filling process (Final Bulk Lot No. CTSARB0120, CTSARB0220, CTSARB0320)

Figure 5

The purity assessment of final bulk and final lot (final stage) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane-1 to 6 represents 0.5ug to 8.0 ug albumin and lane 6: CTSARB0120 (Final bulk) lane: CTSARF0120 (Final lot (stage)). Lane -8: Mol. Wt Marker: (Top to Bottom) – 116, 66.2, 45.0, 35, 25, 18.4 & 14.4 KDa

Figure 6

Evaluation of specificity of antibodies raised in equines against inactivated SARS-CoV-2 virus and comparison of binding of full length IgG in plasma and the F(ab')₂ fragments in purified bulk and final lot vials produced from the plasma

Supplementary Files

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