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Comparison of potency assays to assess SARS-CoV-2 neutralizing antibody capacity in COVID-19 convalescent plasma

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ABSTRACT

Convalescent plasma is plasma collected from individuals after resolution of an infection and the development of antibodies. Passive antibody administration by transfusion of convalescent plasma is currently in clinical evaluations to treat COVID-19 patients. The level of neutralizing antibodies vary among convalescent patients and fast and simple methods to identify suitable plasma donations are needed. We compared three methods to determine the SARS-CoV-2 neutralizing activity of human convalescent plasma: life virus neutralization by plaque reduction assay, a lentiviral vector based pseudotype neutralization assay and a competition ELISA-based surrogate virus neutralization assay (sVNT). Neutralization activity correlated among the different assays; however the sVNT assay was overvaluing the low neutralizing plasma. On the other hand, the sVNT assay required the lowest biosafety level, is fast and is sufficient to identify highly neutralizing plasma samples. Though weakly neutralizing samples were more reliably detected by the more challenging lentiviral vector based assays or virus neutralization assays. Spike receptor binding competition assays are suitable to identify highly neutralizing plasma samples under low biosafety requirements. Detailed analysis of *in vitro* neutralization activity requires more sophisticated methods that have to be performed under higher biosafety levels.

1. Introduction

The ongoing worldwide pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections, is a great threat to global public health. Currently no specific treatment or vaccines are available. Passive immunization by plasma therapy however, has the potential to be used as an emergency treatment. Plasma was applied to humans already in 1917 for the treatment of patients with acute poliomyelitis (Amoss and Chesney, 1917). Thereby, plasma collected from patients who have recovered from the disease, is transfused into acutely infected patients. This treatment has been successfully used as a safe and efficient therapy for infections with the coronaviruses SARS and MERS (Middle East

respiratory coronavirus) and during the Ebola outbreak (Cheng et al., 2005; Ko et al., 2018; Winkler and Koepsell, 2015). Currently studies are ongoing and first successful treatments of COVID-19 patients are being published (Shen et al., 2020; Duan et al., 2020; Ye et al., 2020; Ahn et al., 2020). Apart from finding the right clinical parameters, e.g. stage of disease or symptoms, for plasma transfusion, identifying plasma sample with therapeutic potential is the main challenge for this type of therapy. Patients who have recovered from COVID-19 and have high neutralizing antibody titer are valuable donors. However, the amount of antibodies and the virus neutralizing activity in convalescent serum varies rigorously between patients (Robbiani et al., 2020). The availability of high titer convalescent plasma is less abundant than expected. A recent study of 175 Chinese patients, who recovered from mild COVID-19, described

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that 6 % of the patients did not produce detectable levels of neutralizing antibodies and 30 % of them only very low neutralizing titers (Wu et al., 2020a).

Here, we compared three different assay systems to determine in vitro SARS-CoV-2 neutralizing activity in convalescent plasma to identify a simple and reliable way to define samples with therapeutic potential.

2. Materials and methods

2.1. Cell culture

HEK293T-hACE2 (Glowacka et al., 2010), HEK293 T (ATCC CRL-3216) and Vero E6 cells (ATCC CRL-1586) were cultured at 37 °C under 5 % CO₂ and grown in Dulbecco's modified Eagle medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10 % fetal bovine serum (PAA, Pasching, Austria) and 5 % L-glutamine (200 mM; Lonza, Verviers, Belgium) and 1% penicillin/streptavidin (Fisher Scientific, Schwerte, Germany).

2.2. Plasma samples

Human naïve and SARS-CoV-2 positive plasma was obtained from the German Red Cross from volunteer blood donors. Plasma samples were heat-inactivated at 56 °C for 30 min.

2.3. Virus neutralization assay

Plaque reduction neutralization tests were done as described before (Wölfel et al., 2020). In short, VeroE6 cells (4×10^5 cell/mL) were seeded in 24-well plates the day before. Prior to PRNT patient plasma were heat-inactivated at 56 °C for 30 min and diluted 1:20 up to 1:640. For each dilution step PRNT testing was done in duplicates. For PRNT samples were diluted in OptiPro (Fisher scientific, Schwerte, Germany) and mixed 1:1 with virus solution containing 100 plaque forming units of SARS-CoV-2 (EPI ISL 406862) and incubated at 37 °C for 1 h. The solution was added onto two wells of a 24-well plate. After 1 h at 37 °C the supernatants were discarded, the cells were washed once with PBS and supplemented with 1.2 % Avicel solution in DMEM (Merck, Darmstadt, Germany). After 3 days at 37 °C, the supernatants were removed and the 24-well plates were fixed and inactivated using a 6 % formaldehyde/PBS solution and stained with crystal violet as described (Herzog et al., 2008).

2.4. Pseudotype-based neutralization assay

Lentiviral vectors were prepared in HEK293 T cells by co-transfection using Lipofectamine 2000 (Thermo Fisher, Darmstadt, Germany) as described previously (Henss et al., 2019). Plasmids encoding HIV-1 Gag/pol, rev, the firefly luciferase encoding lentiviral vector genome and the SARS-Cov-2 full-length spike gene (#MN908947) or VSV-G were transfected. Vectors were concentrated by ultracentrifugation and stored at -80 °C. Pseudotyped vectors and serially diluted human plasma/serum (1:60 to 1:14,580) were incubated in triplicates for 30 min. at 37 °C and used to transduce HEK293T-hACE2 cells. After 48 h, luciferase substrate was added to measure luciferase activity. The reciprocal area under the curve (AUC) value calculated for each sample corresponds to the neutralization activity. AUC values were determined using the GraphPad Prism 7.04 software (La Jolla, CA, USA). Mean values and standard deviations were calculated in Excel.

2.5. sVNT assay

The spike protein (S) receptor binding domain (RBD) is responsible for recognizing the cell surface receptor, angiotensin converting enzyme-2 (ACE2). The RBD of SARS-CoV-2 S protein strongly interacts

with hACE2. The SARS-CoV-2 sVNT Kit (Genscript; Leiden, Netherlands) is a blocking ELISA, which mimics this virus receptor binding process. The protein-protein interaction between a horseradish peroxidase (HRP) conjugated recombinant SARS-CoV-2 RBD fragment (HRP-RBD) and hACE2 can be blocked by neutralizing antibodies against the SARS-CoV-2 RBD and residual HRP activity is measured as a surrogate for neutralization. The assay was performed with 1:50 diluted plasma following the manufacturer's instructions.

3. Results

The SARS-Cov-2 neutralizing activity was determined by three different methods. First the gold standard method, virus neutralization assays was performed. Infectious SARS-Cov-2 was used to infect Vero E6 cells in the presence of decreasing amounts of convalescent plasma and the 50 % plaque reduction titer was determined (Fig. 1A). The assay was performed a second time with higher plasma dilutions because for some samples the endpoint titer could not be determined (Fig. 1A in blue) and these values are depicted in orange in Fig. 1A. Several samples (# 2, 4, 5, 6, 16, 20 and 21) showed high neutralization activity above a PRNT50 of 1:350, which is recommended for plasma transfer by the US and European Commission authorities (<https://www.covid19treatmentguidelines.nih.gov/immune-based-therapy/blood-derived-products/convalescent-plasma/> and (EU Ref. Ares(2020)3256185-23/06/2020; Tonn et al., 2020).

Next, the same samples were analyzed with lentiviral vectors pseudotyped with the full-length SARS-CoV-2 spike protein. These vectors acquire the host spectrum of the coronavirus and a rapid read out of the results is possible by the transfer of the luciferase gene, which serves as a measure of infection. Addition of neutralizing antibodies directed against SARS-Cov-2 reduces the luciferase activity. Neutralization activity of plasma was calculated by the area under the curve (AUC) generated by plasma dilutions and their reciprocal values are depicted (Fig. 1B). With this assay, samples 2, 4, 5, 16, 17, 18 and 20 showed the highest neutralization activity.

The third assay is a surrogate neutralization assay and is based on binding of the horseradish peroxidase-labeled SARS-CoV-2 receptor binding domain (RBD) to its receptor angiotensin converting enzyme 2 (ACE2) coated on ELISA plates. Neutralizing antibodies bind to RBD and compete with ACE2 binding. A 20 % inhibition of receptor binding was set as a negative cut-off of the assay. With this assay, samples number 2, 4, 5, 16, 17, 18, 20 and 21, showed high neutralizing activity (Fig. 1C).

The different assay results were plotted against each other and showed linearity with a measure of certainty of $r^2 = 0.79$ for pseudotype against the virus neutralization assay (Fig. 2A). Slightly less linearity was observed for the sVNT and the virus neutralization with $r^2 = 0.68$ (Fig. 2B). However, if the analysis was restricted to highly neutralizing plasma samples and the values of the virus neutralization assay with higher diluted plasma were used, a good correlation of pseudo-neutralization and virus neutralization corresponding to a measure of certainty of $r^2 = 0.84$ was detectable (Fig. 2C). This was less significant for the comparison of the sVNT versus the virus neutralization assay with $r^2 = 0.60$ (Fig. 2D).

In general, the sVNT assay overestimated the samples with low neutralizing activities and was less reliable for these types of plasma samples (Fig. 2B, C and Fig. 1C samples 7, 15, 25, 28, 29). However, highly neutralizing samples could be identified, when using a cut-off of 50 % inhibition.

4. Discussion

Transfusion of convalescent plasma might be a therapeutic option for the treatment of COVID-19, but the identification of suitable plasma donors is hampered by the substantially variable levels of neutralizing antibodies in convalescent patients. Therefore, we evaluated here, which assay would be suitable to identify donors.

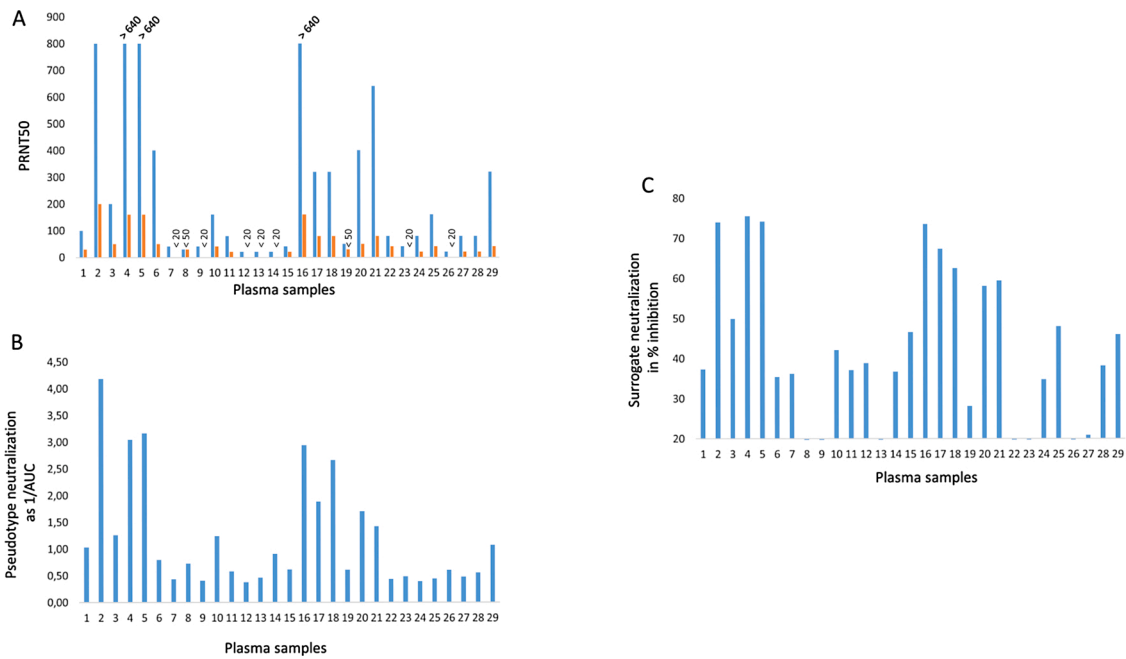


Fig. 1. In vitro SARS-CoV-2 neutralization activity of human plasma samples.

Human plasma samples were analyzed by three assays. (A) Virus neutralization, which was done with two different dilution series of plasma. Lower dilutions are depicted in blue, higher dilution depicted in orange. SARS-CoV-2 neutralization is shown as plaque reduction neutralization titers 50 (PRNT50), which correspond to the plasma dilution that reduces the virus plaques by 50 %. Samples with titers >640 are indicated. (B) Pseudotyped vector neutralization is given as the reciprocal value of the area under the curve (AUC) obtained as relative light units with plasma dilutions. (C) The surrogate virus neutralization assay sVNT shows neutralization as inhibition of HRP-coupled RBD binding to the ACE2 receptor. Plasma samples were diluted 1:50 for the assay.

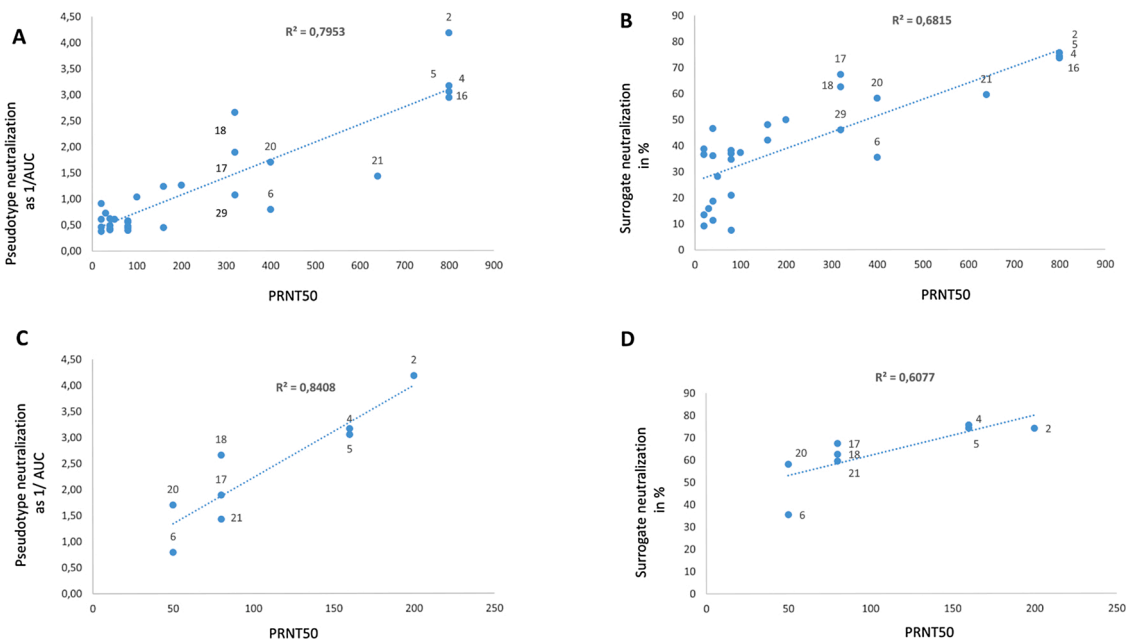


Fig. 2. Comparison of assays with the virus neutralization assay.

(A) Pseudotype neutralization was plotted against the virus neutralization activities. The r^2 value indicates the certainty of the values to be at the trend line and shows with $r^2 = 0.79$ comparability of the two assays. (B) sVNT assay values were plotted against the virus neutralization. The $r^2 = 0.68$ shows less comparability of the two assays compared to A. (C) Pseudotype neutralization activities of highly neutralizing plasma samples were plotted against the virus neutralization activity. The r^2 value indicates the certainty of the values to be at the trend line and shows with $r^2 = 0.84$ high comparability of the two assays. (D) The sVNT activities of highly neutralizing plasma samples were plotted against the virus neutralization activity. The r^2 value of 0.6 indicates less comparability of the two assays compared to C.

The three assays used are able to identify highly neutralizing plasma, but they require different biosafety levels to perform the assay. The virus neutralization has to be carried out under biosafety 3 level, the

pseudotype neutralization assay requires biosafety level 2 and sVNT assay is a simple ELISA, which can be performed in any laboratory without special biosafety requirements. In addition, the pseudotype

assay and the virus neutralization assay are time consuming and require 2 or 3 days respectively, until results are available. The sVNT assay can be performed in 2–3 h. However, the sVNT assay overestimated the samples with low neutralizing activities, but increasing the cut-off to 50 % inhibition enabled the identification of highly neutralizing samples. Therefore, the sVNT assay, although not yet approved for diagnostic application, is superior for a clinical setting and allows the fast screening of convalescent plasma samples in low biosafety level laboratories. Also other ELISA-based sVNT assay systems have been developed recently (Bošnjak et al., 2020; Abe et al., 2020; Ding et al., 2020) and different kits are commercially available. Assay comparisons were performed by multiple groups and overall, the sVNT assays correlated well with the other neutralization assays (Abe et al., 2020; Meyer et al., 2020; Bond et al., 2020; McGregor et al., 2020; Bošnjak et al., 2020; Ding et al., 2020). The sVNT assays are well suited for a rapid prescreening of patient plasma to identify donors with high neutralization activity. In addition, sVNT kits are useful to analyzed animal sera from preclinical vaccine studies, because no adaptation of the assay is required (Perera et al., 2020). A general limitation of these assays is that they are only able to detect neutralizing antibodies that function by blocking the interaction between the RBD and ACE2, although most neutralizing antibodies fulfill this requirement, single antibodies have been described that use other mechanisms for neutralization (Wang et al., 2020; Wu et al., 2020b).

Detailed analysis of recipients of the plasma donation might require the more sophisticated assays. For the clinical evaluation of this therapeutic option, also the recipients have to be tested because they might already have highly neutralizing antibodies and additional antibody treatment might not help (Arvind Gharbharan et al., 2020). These more detailed analyses of SARS-CoV-2 neutralization activity require either, virus neutralization or pseudotype assays. In addition, basic research on the induction of SARS-CoV-2 immune responses should be performed with these assays. Both assays are comparable and a direct calibration of two assays will be possible when an international standard is available.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRedit authorship contribution statement

Christine von Rhein: Data curation, Formal analysis, Investigation, Methodology. **Tatjana Scholz:** Data curation, Formal analysis, Investigation, Methodology. **Lisa Henss:** Data curation, Formal analysis, Investigation, Methodology. **Romy Kronstein-Wiedemann:** Data curation, Formal analysis, Investigation, Methodology. **Tatjana Schwarz:** Data curation, Formal analysis, Investigation, Methodology. **Roman N. Rodionov:** Data curation, Formal analysis, Investigation, Methodology. **Victor M. Corman:** Data curation, Formal analysis, Investigation, Methodology. **Torsten Tonn:** Data curation, Formal analysis, Investigation, Methodology. **Barbara S. Schnierle:** Data curation, Formal analysis, Investigation, Methodology.

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