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Application of the SARS-CoV-2-S1 ACE-2 receptor interaction as the basis of the fully automated assay to detect neutralizing SARS-CoV-2-S1 antibodies in blood samples

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ABSTRACT

A quantitative, high throughput, fully automated diagnostic method for the detection of neutralizing anti-SARS-CoV-2 antibodies was developed on the Phadia system based on the interaction of SARS-CoV-2 S1 protein and the human ACE-2 receptor. This method was compared to the current state of the art plaque reduction neutralization test (PRNT) and a high correlation between the two methods was observed. Using a large cohort of blood samples from convalescent patients and controls the method displays very high sensitivity and specificity (99.8% and 99.99%, respectively). Neutralizing antibody titers of mRNA-1273 and BNT162b2-vaccinated persons can also be quantified with this method as well. This fully automated method provides the possibility to determine anti-SARS-CoV-2 neutralizing antibody concentrations in just 2 h.

1. Introduction

The SARS-CoV-2 coronavirus 2019 (COVID-19) infection leads to severe respiratory symptoms and life-threatening conditions with a high mortality rate (Zhou et al., 2020). The SARS-CoV-2 virus uses its spike protein for the cell entry, which is mediated by the binding of S1 as part of the spike proteins via its receptor binding domain (RBD) to angiotensin-converting enzyme 2 (ACE-2) receptor on the surface of the epithelial cells of the respiratory tract (Lan et al., 2020; Walls et al., 2020). This interaction can be impaired by neutralizing antibodies that bind to the RBD within its ACE-2 receptor binding interface and thus sterically hinder the binding of the two proteins. This principle was shown to be applied in assays to detect SARS-CoV-2 neutralizing antibodies in ELISA format (Taylor et al., 2021; Tan et al., 2020).

In fact, those neutralizing antibodies can be used as treatment in the early phase of infection to prevent patients at risk from developing severe symptoms (Zost et al., 2020; Jiang et al., 2020; Deb et al., 2021). Various studies showed a strong antibody titer decrease reaching even the seronegative state 6–12 months post infection after second vaccination (Khoury et al., 2021; Wheatley et al., 2021; Doria-Rose, 2021; Padoan et al., 2022; Vicenti et al., 2021). Thus, there is an urgent need of tests quantifying the neutralization potential in samples to determine if the persons need to be revaccinated after 6–12 months to be safe from a COVID-19 reinfection. Furthermore, this assay should be able to be used as a tool for the characterization of neutralization potential of antibody preparations against wildtype and mutant viruses as well.

The state-of-the-art method of determining the neutralization potential of samples is the plaque reduction and neutralization test (PRNT). In this method, Vero E6 cells are infected with the SARS-CoV-2 coronavirus in the presence of diluted serum or plasma samples of convalescent patients or vaccinated individuals. Infection of Vero E6 cells with SARS-CoV-2 leads to the cytopathic effect (CPE), which is visible under the light microscope. The NT50 is defined as sample dilution, at which CPE is prevented by 50%. NT50-values are calculated and used as a quantitative value of samples’ neutralization potential. This assay is laborious and needs long incubation times resulting in a week to get the required NT50-values. In addition, the PRNT requires a biosafety level 3 laboratory (Bewley et al., 2021).

As an alternative to the biosafety level 3 PRNT, the so called...
pseudovirus-based neutralization test is available. This assay uses recombinant virus expressing SARS-CoV-2 Spike protein instead of the SARS-CoV-2 itself. The pseudo-virus-based neutralization test as a surrogate of the PRNT can be performed in biosafety level 2 laboratories, but still requires the use of live viruses and cells thus being tedious and time-consuming. In addition, those assays are hardly automatable, making it impossible to use them in the clinical routine diagnostics (Nie et al., 2020).

To develop an assay to identify neutralizing antibodies for the clinical routine testing, a high throughput screening system is needed. As such, the Phadia system with its fully automated instruments with different sample capacity is an optimal platform to measure neutralizing such, the Phadia system with its fully automated instruments with time-consuming. In addition, those assays are hardly automatable, but still requires the use of live viruses and cells thus being tedious and SARS-CoV-2 itself. The pseudo-virus-based neutralization test as a surrogate.

Here, we present a fully automated, high throughput assay showing a high correlation to the PRNT and being able to detect neutralizing antibodies in samples of convalescent and vaccinated persons sensitively and with a high specificity.

2. Material and methods

2.1. Samples

2.1.1. Correlation to PRNT

For the correlation of the ACE-2 receptor binding inhibition assay to PRNT, 37 serum and plasma samples from PCR-proven COVID-19 convalescent individuals with PRNT50 values determined in the PRNT by TexCell (Eyr, France) and 44 pre-pandemic blood donor samples were tested. VERO E6 cells were infected with the SARS-CoV-2, isolate: 2019-nCoV/Italy INM1 2nd P VERO E6 11.02.2020, in the presence of sample dilutions in 8 replicates in 96-well cell culture plates. The cytopathic effect (CPE) was read on 6th day post-infection. The neutralization titer 50 (NT50) corresponding to the sample dilution which prevents cells from CPE in 50% of replicates was calculated according to Spearman-Kärber formula.

2.1.2. Correlation to S1 IgG

For determination of sensitivity 637 serum and plasma samples of COVID-19 convalescent persons with PCR-proven infection with 4–27 weeks post-PCR-positivity and a control cohort consisting of 158 pre-pandemic samples from different infectious diseases (Adenovirus, EBV, Enterovirus, Haemophilus influenzae, infection, Measles, Mumps, Respiratory Syncytial Virus, Variella Zoster Virus, HCoV-229E, HCoV-HKU1, HCoV-NL63, HCoV-OC43, Parainfluenza, Influenza, Mycoplasma pneumoniae, Parvo B12), as well as blood donors were used.

2.1.3. Comparison of vaccinated and COVID-19-convalescent samples

For the comparison of neutralizing antibody titers of vaccinated and COVID-19 convalescent persons, serum and plasma samples of 1–3 weeks post-PCR-positivity from COVID-19 infected persons and samples of persons 1–3 weeks after second vaccination with mRNA-1273 (Moderna) (16 samples), and BNT162b2 (Pfizer) (18 samples) were used.

2.2. Proteins

SARS-CoV-2. Spike S1 protein, NCBI Reference Sequence: YP_009724390.1 with amino acids 14–681, ACE-2 receptor proteins and the neutralizing antibody, clone 6G7 were obtained from Icosagen AS (Tartu, Estonia).

2.3. ACE-2 binding inhibition assay

There is a high degree of conformance between different Phadia instruments, which was shown for 31 FDA-cleared assays as exemplified by the 510(k) for the EliA anti-cyclic citrullinated peptides (CCP) (Mann, 2006). As such, Phadia-100 and Phadia-250 results are assumed to be equivalent in terms of correlation and precision and the instruments were chosen only depending on the required experimental scale.

For the assay described in this paper, the fully automated, high throughput Phadia platform, in particular, Phadia-250 instrument, was used (Villalta et al., 2002). N-hydroxysuccinimide ester activated biotin was used for covalent coupling of biotin to primary amino groups (−NH2) of the SARS-CoV-2 spike protein (Icosagen AS, Tartu, Estonia). Biotinylated SARS-CoV-2 S1 protein was immobilized on streptavidin-coated microcavities at a concentration of 0.2 μg/ml in 1×PBS as buffer and Stabilcoat (Surmodics) as blocking and stabilizing agent. ACE-2 receptor (Icosagen AS, Tartu, Estonia) was coupled to β-galactosidase (Thermo Fisher Scientific, Uppsala, Sweden) using LC-SPDP (succinimidyl 6-(3(2-pyridyldithio)propionamido)hexanoate) (Thermo Scientific, Rockford, Illinois, U.S.A.) as cross-linker. The conjugate was purified on a Superose 6 column (Cytiva, Uppsala, Sweden) to remove unconjugated ACE-2 and β-galactosidase and used as assay-conjugate at a concentration of 0.425 μg/ml in EliA conjugate buffer. Microcavities and the conjugate were used in the Phadia instrument with the commercially available set of Phadia system reagents including EliA Sample Diluent, washing buffer, 4-methylumbelliferyl-β-D-galactopyranoside as substrate and stop solution.

As calibrator material, recombinant monoclonal human neutralizing antibody, whose genetic material was isolated from PBMC of a convalescent person (Clone 6G7, from Icosagen AS, Tartu, Estonia) was used in concentrations of 0–5 μg/ml. The calibration curve was calculated with a Rodbard 4-parameter fit using Excel and Graphpad.

For the correlation to PRNT and S1 IgG, samples were measured in 1:2 instrument dilution.

For other studies, dilutions were chosen to obtain a signal within the dynamic range of the calibration curve. Concentration of neutralization antibodies in μg/ml was calculated considering the applied sample dilution.

2.3.1. Intra- and Inter-assay precision

To determine the intra and inter-assay precision of the ACE-2 receptor binding inhibition assay, the variability was assessed in a study evaluating 4 samples in 5 independent runs with a total of 100 replicates. The evaluation was carried out on a Phadia 100 instrument. A calibration curve was included in each run. Both intra- and inter-assay precision were characterized by calculating the mean and coefficients of variation (CV%) using Analyze-It Microsoft add-in program.

2.3.2. S1 IgG FEIA

S1 IgG fluorescence enzyme immune assay (FEIA) from Thermo Scientific Sp1 IgG Test was used on fully automated Phadia instruments according to the manufacturer’s instruction.

3. Results

3.1. Assay development

To detect neutralizing antibodies, the interaction between the SARS-CoV-2 Spike protein and human ACE-2 receptor was used. Biotinylated, recombinant SARS-CoV-S1 protein was immobilized onto streptavidin-coated EliA microcavities. As conjugate, recombinant human ACE-2 receptor was coupled to β-galactosidase using crosslinker technology (Fig. 1). The assay is built as a competitive assay. In absence of SARS-CoV-2 neutralizing antibodies the conjugate binds to the RBD of the immobilized SARS-CoV-S1 protein resulting in a high fluorescence signal as consequence of the cleavage of 4-methylumbelliferyl-β-D-galactopyranoside as substrate (Fig. 1 A). If RBD-binding neutralizing antibodies are present, they bind to the RBD on the solid phase at the ACE-2 receptor binding interface, thus sterically hindering the ACE-2...
receptor binding resulting in low assay signal (Fig. 1 B). Fig. 2 A shows low signals of samples from COVID-19 convalescent persons in contrast to pre-pandemic blood donors or the sample diluent as control.

To quantify the neutralizing antibodies in samples (Fig. 2 B), a recombinant antibody at a concentration of 0–5 μg/ml could be established as calibrator.

To further characterize the ACE-2 receptor binding inhibition assay intra- and inter-assay precision was determined. The corresponding CV % values for the intra-assay precision were between 3.1 and 4.5%, and between 4.6 and 5.7% for the inter-assay precision confirming high reproducibility of the assay (Table 1).
3.3. Determination of specificity and comparison of sensitivity to the Phadia S1 IgG assay in convalescent COVID-19 samples

After having shown a good correlation between the ACE-2 receptor binding inhibition assay and PRNT, we aimed to determine the specificity and to compare the sensitivity of the ACE-2 binding inhibition assay to the Phadia S1 IgG assay in convalescent COVID-19 samples. Therefore, a cohort of 637 samples of COVID-19 convalescent patients with proven PCR-positivity was tested. Pre-pandemic-sourced blood samples of respiratory and other infectious disease-patients, as well as blood donors served as controls. The cohort also contained samples from common cold corona viruses infected individuals: HCoV-229ECorona, HCoV-HKU1, HCoV-NL63, HCoV-OC43. All sera were tested for SARS-CoV-2 IgG antibodies using the FEIA-based SARS-CoV-2 S1 IgG assay from Thermo Fisher Scientific (Mylemans et al., 2021) on the fully automated Phadia platform.

In the ACE-2 binding inhibition assay, the concentrations of samples from COVID-19 convalescent individuals in a dilution of 1:2 ranged between 0.2 and 5.0 \( \mu \text{g/ml} \) with 167 samples above the measuring range. The concentrations of control samples measured at the same dilution ranged between 0 and 0.2 \( \mu \text{g/ml} \) (Fig. 4 A). The mean of signals for COVID-19 convalescent samples was 2.8 ± 1.7 \( \mu \text{g/ml} \) and for control samples 0.03 ± 0.05 \( \mu \text{g/ml} \), respectively, suggesting a good discriminatory ability between the two sample types. In fact, there was only a small signal overlap between the control samples and samples of COVID-19 convalescent persons. This leads to only seven samples out of 637 which could not be discriminated from the controls.

To assure a high specificity of 99.99% (95% CI = 97.6% to 99.9%) the threshold positivity value of 0.3 \( \mu \text{g/ml} \) was set. With this cut-off value a sensitivity of 98.9% (95% CI = 97.7% to 99.5%) was determined for this sample cohort.

In contrast to the ACE-2 receptor binding inhibition assay, the S1 IgG
Among these two samples, one is even above the manufacturer’s cut-off (10 U/ml) a sensitivity of 86.2% (95% CI = 83.3 to 88.6% and a specificity of 99.4% (95% CI = 96.5% to 99.9) was determined for this sample cohort.

To properly compare the two assays, sensitivity was addressed at the fixed specificity of 99%. The sensitivity at specificity of 99%, was 98.9% (95% CI = 99.1% to 100%) and 95% (95% CI = 93.0% to 96.4%) for the ACE-2 binding inhibition assay and the Phadia S1 IgG assay, respectively. This analysis suggests that the ACE-2 receptor binding inhibition assay is more specific than the S1 IgG assay. Indeed, the two highest samples from the pre-pandemic control cohort show S1 IgG signals of 12.8 and 7.2 μg/ml, respectively. One sample corresponds to a blood donor and the other one is Influenza A IgG positive. Among these two samples, one is even above the manufacturer’s cut-off value and the other one is in the borderline zone. In contrast, both samples display concentrations of 0.15 and 0.09 μg/ml in the ACE-2 receptor binding inhibition assay, being thus clearly below the positivity threshold of 0.3 μg/ml.

In summary, the developed, fully automated ACE-2 receptor binding inhibition assay displays high sensitivity and specificity and can be used to quantify SARS-CoV-2 neutralizing antibodies in samples of COVID-19 convalescent persons. This assay offers even higher sensitivity than the commercially available S1 IgG used on the same Phadia platform.

3.4. Quantification of neutralizing antibodies in samples of vaccinated persons

So far, the ACE-2 receptor binding inhibition assay proved to be able to quantify the SARS-CoV-2 neutralizing antibodies in samples of COVID-19 convalescent persons.

Along with worldwide SARS-CoV-2 vaccination, the need to quantify the neutralizing antibodies as a result of vaccination might become necessary. Assuming that neutralizing antibody titers from a vaccination could fade over time (Doria-Rose, 2021), it could become necessary to determine the neutralization antibody concentration to decide about need of revaccination. This would require population wide tests for neutralizing antibodies, which cannot be fulfilled using PRNT, which can be performed with single samples only. For population-wide tests fully automated assays on scalable systems, such as ACE-2 receptor binding inhibition assay on a Phadia system, would be needed. For this purpose, we aimed to quantify neutralizing antibodies in samples of mRNA-1273-, and BNT162b2-vaccinated persons (Baden et al., 2021; Polack et al., 2020) and to compare these titers with those in samples of COVID-19 convalescent persons. As antibodies have been reliably detected 1–3 weeks following recovery from COVID-19, or after the second vaccine dose, we chose to analyze samples at these time points. Neutralizing antibody titers of 30 samples of COVID-19 convalescent persons were compared to 16 samples of mRNA-1273-vaccinated and 18 samples of BNT162b2vaccinated persons yielding a mean signal of 58.63 μg/ml (95% CI = 43.46 to 73.79 μg/ml), 291.1 μg/ml (95% CI = 202.7 to 379.4 μg/ml and 113.8 μg/ml (95% CI = 61.79 to 165.8 μg/ml) (Fig. 5) and were clearly elevated compared to signals of pre-pandemic blood donors. ANOVA shows that there is a significant difference between neutralizing antibody titers of COVID-19 convalescent and mRNA-1273-vaccinated persons (Table 2). This difference might be accounted by cohort differences, since COVID-19 convalescent persons were significantly older (mean age 69) with harsh symptoms of dyspnea and being hospitalized compared to mRNA-1273-vaccinated persons.

Table 2

| Multiple comparison                          | Adjusted P value |
|----------------------------------------------|------------------|
| COVID-19 convalescent vs. mRNA-1273-vaccinated| 0.0094           |
| COVID-19 convalescent vs. BNT162b2 mRNA-vaccinated | >0.9999         |
| COVID-19 convalescent vs. pre-pandemic blood donors | <0.0001         |
| mRNA-1273-vaccinated vs. BNT162b2 mRNA-vaccinated | 0.5799           |
| mRNA-1273-vaccinated vs. pre-pandemic blood donors | <0.0001         |
| BNT162b2 mRNA-vaccinated vs. pre-pandemic blood donors | <0.0001         |

Fig. 5. Comparison of neutralization antibody concentrations in samples of COVID-19 convalescent persons, mRNA-1273- and BNT162b2-vaccinated persons 1–3 weeks post PCR positivity or post second vaccination dose. Means for the samples of COVID19-convalescent, mRNA-1273- and BNT162b2-vaccinated persons are 58.63, μg/ml (95% CI = 43.46 to 73.79 μg/ml) 291.1 μg/ml (95% CI = 202.7 to 379.4 μg/ml) and 113.8 μg/ml (95% CI = 61.79 to 165.8 μg/ml), respectively.
with mean age of 41.

In summary, the newly developed ACE-2 receptor binding inhibition assay is able to quantify neutralizing antibody concentrations not only in samples of COVID-19 convalescent persons but also in samples of vaccinated individuals as exemplified here with samples from mRNA-1273- and BNT162b2-vaccinated persons 1–3 weeks after the second vaccination.

4. Discussion

So far, assays with ACE-2 receptor immobilized on solid phases and RBD conjugated to reporter enzymes were correlated to PRNT. This assay format requires a preincubation step of sample with the enzyme conjugated RBD before this mixture can be applied to the solid phase (Taylor et al., 2021; Tan et al., 2020). This preincubation step would be problematic in the pipetting sequence of fully automated instruments, because most instruments perform the transfer of sample to the solid phase as the first pipetting step and would require either preincubation outside of the instrument or the incubation of sample-conjugate mixture directly on the solid phase. To overcome this obstacle SARS-CoV-2 S1 protein was immobilized on the solid phase and ACE-2-receptor bound to the reporter enzyme was used as conjugate. This setup fits better into the standard pipetting sequence of Phadia instruments without the need of a preincubation step outside of the instrument.

The ACE-2 receptor binding inhibition assay shows a very good agreement with PRNT in COVID-convalescent and pre-pandemic blood donor samples. In PRNT, ca. half of pre-pandemic donor samples displayed cell toxicity at 1:16 sample dilution, which interfered with the CPE analysis, and is a disadvantage of this cell-based assay in comparison to the ACE-2 receptor binding inhibition assay.

The weakness of the ACE-2-receptor binding inhibition assay is the focus on the interaction between S1 and ACE-2 receptor only. Undoubtedly, this interaction is the main mechanism for the binding of the SARS-CoV-2 to the epithelial cells. But there are other mechanisms, that might be involved in the cell entry of the virus as well. It was shown that the virus binds to the heparan sulfate on the cell surface via its RBD (Baden et al., 2021). Another neglected aspect by the ACE-2 receptor binding inhibition assay is the value of the N-terminal domain of the spike protein (Polack et al., 2020), which is a target of some neutralizing antibodies, thus playing a significant role in virus transmission (Israel et al., 2021).

Considering, that the ACE-2 receptor binding inhibition assay only focuses on the RBD ACE-2-receptor interaction, this assay displays a high correlation with PRNT. This suggests that the RBD ACE-2 receptor interaction is the crucial step of the SARS-CoV-2 infection thus mimicking the viral infection at the cellular level very well.

By comparing the ACE-2 binding inhibition assay with the Phadia S1 IgG enzyme immunoassay based on the same fully automated Phadia platform, it was found that the test even surpassed the high specificity proven by the commercial EliA SARS-CoV2 Sp1 IgG test. It seems that by focusing on the ACE2 receptor RBD interaction one would be able to reduce the impact of non-specific antibody binding to the solid phase significantly, which is a big strength of the assay (Sahin et al., 2020).

Using the samples of mRNA-1273- and BNT162b2-vaccinated persons we could show, that the ACE-2 receptor binding inhibition assay is able to quantify neutralizing antibodies in samples of vaccinated persons as well. The concentrations are rather high, so a higher sample dilution than the one used for the measurement of COVID-19 convalescent samples is required for most of samples. It seems that the neutralizing antibody titers in samples of mRNA-1273-, and BNT162b2-vaccinated persons are higher than in the samples of COVID-19 convalescent persons having roughly the same time interval of 1–3 weeks after the last contact with either virus or vaccine as immunogen, which is in line with the current data from the literature (Israel et al., 2021; Sahin et al., 2020).

The data presented here are measured with the assay built with the biotinylated SARS-CoV-2 S1 protein of the Wuhan virus strain and operated on Phadia-250 and Phadia-100 instruments. After several infection waves with the alpha, beta, delta and currently omicron variant, one might conclude, that the assay would be outdated. But, the application of biotinylated SARS-CoV-2 S1 protein on the solid phase provides the possibility to change the antigen according to current virus strain, making the assay easy to adapt to future infection waves. In addition, the application of the assay on the Phadia™ system with Phadia-200, Phadia-250 and Phadia-5000 instruments provides the unique fully automated assay of different sample scales to date.

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Declaration of Competing Interest

All authors are employees of Thermo Fisher Scientific ImmunoDiagnostics Phadia GmbH.

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