Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Development and characterization of an indirect ELISA to detect SARS-CoV-2 spike protein-specific antibodies

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ABSTRACT

The current *Severe acute respiratory syndrome-related coronavirus 2* (SARS-CoV-2) pandemic is a public health emergency of international concern. Sensitive and precise diagnostic tools are urgently needed. In this study, we developed a SARS-CoV-2 spike (S1) protein enzyme-linked immunosorbent assay (ELISA) to detect SARS-CoV-2-specific antibodies. The SARS-CoV-2 S1 ELISA was found to be specific [97.8% (95% CI, 96.7% - 98.5%)], reproducible and precise (intra-assay coefficient of variability (CV) 5.3%, inter-assay CV 7.9%). A standard curve and the interpolation of arbitrary ELISA units per milliliter served to reduce the variability between different tests and operators. Cross-reactivity to other human coronaviruses was addressed by using sera positive for MERS-CoV- and hCoV HKU1-specific antibodies. Monitoring antibody development in various samples of twenty-three and single samples of twenty-nine coronavirus disease 2019 (COVID−19) patients revealed seroconversion and neutralizing antibodies against authentic SARS-CoV-2 in all cases. The comparison of the SARS-CoV-2 (S1) ELISA with a commercially available assay showed a better sensitivity for the in-house ELISA.

The results demonstrate a high reproducibility, specificity and sensitivity of the newly developed ELISA, which is suitable for the detection of SARS-CoV-2 S1 protein-specific antibody responses.

1. Introduction

The current *Severe acute respiratory syndrome-related coronavirus 2* (SARS-CoV-2) outbreak was declared a public health emergency of international concern on 30th January 2020 (World Health Organization (WHO), 2020a) and classified as a pandemic on 12th of March 2020 (World Health Organization (WHO), 2020b). In summer 2020, neither specific drugs nor vaccines for the treatment or prevention of COVID-19, the disease caused by SARS-CoV-2, were available. Therefore, rapid diagnosis of COVID-19 cases was mandatory to contain the pandemic. Furthermore, to better understand the development of the current pandemic especially the approximate total number of COVID-19 cases, well characterized serological assays are needed.

Several commercial and in-house serological assays were established for the detection of antibodies against SARS-CoV-2 that are based on the recombinantly expressed viral proteins N, S or truncated versions of the S protein (Xiang et al., 2020; Zhang et al., 2020; Stadlbauer et al., 2020; Okba et al., 2020; Guo et al., 2020; Liu et al., 2020; Traugott et al., 2020; Krüttgen et al., 2020; Kohmer et al., 2020a; Kohmer et al., 2020b). Although different assays are used, the studies comparing different viral antigens in similar assays suggested that the S1 of the surface spike protein is a suitable antigen for SARS-CoV-2 diagnostics (Okba et al.,

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2020; Liu et al., 2020). Another advantage of an S-specific ELISA is the detection of immune responses from vaccinated people since most vaccine candidates express the S protein of SARS-CoV-2. Since several studies found S-specific IgM and IgG antibodies in sera from COVID-19 patients in the same serum samples (Liu et al., 2020; Sun et al., 2020; Haveri et al., 2020), it appears that S-specific IgG antibodies can be detected almost simultaneously to S-specific IgM. The potential cross-reactivity with antibodies against other human CoV (hCoV) can be a challenge when developing a CoV-specific ELISA, as six other hCoV are known to date. SARS-CoV-2 was classified in the *Orthocoronavirinae* subfamily (order *Nidovirales*, family *Coronaviridae*). This subfamily is divided into four genera: *alpha*, *beta*, *gamma* and *deltacoronaviruses*. SARS-CoV-2, like SARS-CoV, MERS-CoV, hCoV OC43 and HKU1, belongs to the *betacoronaviruses*. In addition to these four hCoVs, there are two more: hCoV 229E, hCoV NL63, both of which are *alphacoronaviruses*. Of these viruses, SARS-CoV is the closest relative to the newly emerged SARS-CoV-2. Both SARS-CoV and SARS-CoV-2 as well as hCoV NL63 use angiotensin converting enzyme 2 (ACE2) as the main receptor for virus entry into the host cell (Hulswit et al., 2016; Shereen et al., 2020).

In the present study we describe the development and characterization of an indirect ELISA using the S1 domain of the spike protein to detect IgG antibodies against SARS-CoV-2. The ELISA described here is intended to be used for the detection of immune responses from infected and vaccinated individuals, which is why the spike protein was used as the antigen.

2. Materials & methods

2.1. Cell culture and antibodies

Vero C1008 (ATCC CRL-1586) and HuH7 cells (fully matching the STR reference profile of HuH-7) were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS), penicillin (50 U/mL), streptomycin (50 µg/mL) (P/S) and glutamine (2 mM) (Q). Both cell lines were authenticated by DNA profiling of eight highly polymorphic regions of short tandem repeats in 2016 by the "Leibniz-Institut DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) GmbH". A rabbit polyclonal anti-HKU1 spike protein antibody (Sino Biological, 40021-T60) was used to detect HKU1 S protein. Polyclonal secondary antibodies were purchased from Dako (Dako, Denmark) and used at a dilution of 1:1,000 (ELISA) or 1:40,000 (Western blot).

2.2. Human sera or plasma samples

A total of 120 European human sera from healthy anonymous donors were used to characterize the specificity of the test (collected 2014, Institute of Virology, Philipps University of Marburg). Serum from a healthy voluntary donor (Institute of Virology, Philipps University of Marburg) was used as negative control. Plasma samples of five COVID-19 patients (#11 to #15) were obtained from the Lab of F. Klein (Institute of Virology, Cologne). A detailed description of the course of infection in COVID-19 patients is published elsewhere (patients #11 to #14 correspond to #10, #3, #14 and #7 described by Wölfel et al. (2020); patient #15 corresponds to IDFnC1 described by Kreer et al. (2020).Two of these plasma samples were analyzed and one of them was used as a set of calibrator solutions (standards) in the developed SARS-CoV-2 S1 ELISA. Serial serum samples of eighteen and single serum samples of 29 COVID-19 patients were obtained from the "Zentrum für In-Vitro-Diagnostik – Infektionsdiagnostik (ZIVD), Universitätsklinikum Gießen und Marburg (UKGM)" (#1 to #10; #16 to #23). These samples were collected in the framework of the COVID-19 biomarker study. The study was carried out in accordance with the Declaration of Helsinki and the guidelines of the International Conference for Harmonization for Good Clinical Practice. Ethical approval for the COVID-19 biomarker study was received from the institutional review board at Philipps University Marburg, and patients were enrolled after obtaining informed consent. To further characterize the specificity of the SARS-CoV-2 S1 ELISA 20 sera of MVA-MERS-CoV-S-vaccinated individuals (Koch et al., 2020) and 27 sera of healthy individuals reacting on HKU1 S1 antigen were used. Informed consents to use serum and plasma for scientific research were obtained.

2.3. SARS-CoV-2 S1 ELISA (IgG)

High binding single-break strip microtiter plates (Greiner bio-one, Cat.No.705074) were coated with SARS-CoV-2 spike (S1) protein (Sino Biological, 40, 591-V08H) diluted to 1 µg/ml in sterile phosphate buffered saline (PBS) and incubated for 20 h (+/− 30 min) at 4 °C. Further incubations were performed at room temperature (RT). ELISA plates were washed three times with PBS/0.1% Tween®20 (PBST), and then blocked for 45 min with PBS containing 5% milk powder. Washing procedure was repeated three times with PBST. Human sera were diluted 1:101 in PBST containing 1% milk powder and allowed to react with the S1 protein for 1 h. To confirm reliability and repeatability a blank, a negative control and a set of standards was used on each plate. Six standard solutions were prepared by serial dilution at the basis of 2 of a COVID-19 patient plasma. The undiluted plasma and five consecutive two-fold serial dilutions of the plasma made in PBS with 0.5% BSA were stored in small aliquots at -80 °C as standard solutions S1 to S6. Upon thawing each solution was diluted 1:10 to obtain the final standard solution (S1 = 1:10; S2 = 1:20; S3 = 1:40; S4 = 1:80; S5 = 1:160 and S6 = 1:320). These solutions were further diluted 1:101 as part of the ELISA assay, just as any other sample to be tested. 4-parameter logistic (4PL) regression analysis (software: GraphPad Prism 8) of the standard curve was used to calculate arbitrary ELISA units/milliliter (AEU/ml) for the positive samples. The lowest standard (S6) was set to 100 AEUs/ml, and the highest (S1) was set to 3200 AEU/ml. Samples that exceed the OD value of the highest standard S1 are assigned >3200 AEU/ml. After washing the plates three times with PBST (anthos fluido, ELISA plate washer), polyclonal rabbit anti-human IgG/HRP antibodies (DAKO; P0214) were used for detection (dilution 1:1000, 30 min of incubation). Following another round of washing (two times with PBST, and two times with PBS), 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (SureBlue™ TMB Microwell Peroxidase Substrate, KPL Inc.) were added to each well, and allowed to react for 10 min protected from light. The reaction was stopped with 100 µl/well of TMB-Stop Solution (KPL Inc., Maryland), and the optical density (OD) was determined at 450 nm – 620 nm using an automated spectrophotometer (PHOmo, Autobio Labtec Instruments Co., Ltd.) within 5 min.

2.4. EUROIMMUN SARS-CoV-2 S1 IgG and IgA ELISAs

The respective ELISAs (order number: EI 2606–9601 A or EI 2606–9601 G) were performed according to the manufacturer's instructions using the BEP® (Behring ELISA Processor) III system located at the ZIVD, UKGM. Serum and plasma samples were analyzed at a dilution of 1:101.

2.5. Determination of key performance indicators of SARS-CoV-2 S1 ELISA

The coefficient of variation (CV) is the ratio of the standard deviation (σ) to the mean (µ): [CV = σ/µ]. Intra-assay variability was determined by testing three different positive plasma samples five times within the same assay in six independent assays. Inter-assay variability was determined by evaluating eight different plasma samples and one serum sample in six different assay runs, and by three different operators. In both cases, the results were obtained by using the following formula: [% CV = (σ/mean) × 100]. In each assay, an internal set of standards and a negative control was used to validate the assay and to calculate AEU/ml.

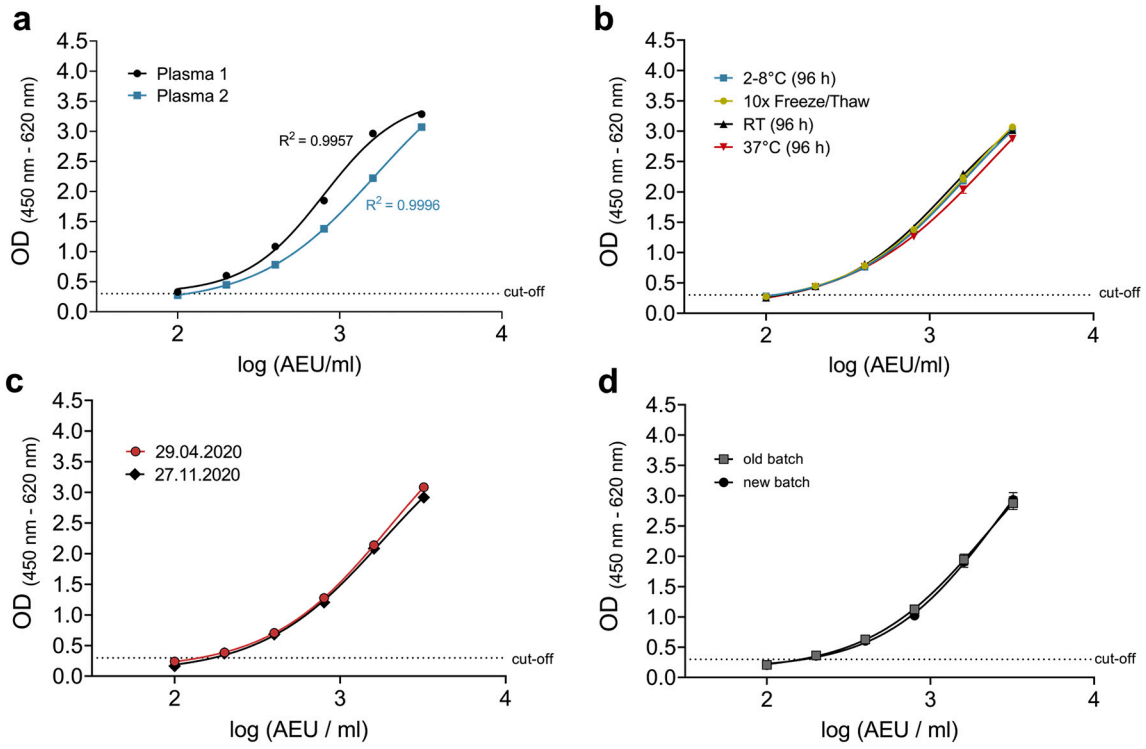

Fig. 1. Standard curve of the SARS-CoV-2 S1 ELISA a Standards (S1–S6) were prepared by two-fold serial dilutions of two human convalescent plasma samples starting at a dilution of 1:1010. A standard curve was created by 4PL regression analysis. The lowest standard (S6) was set to 100 AEUs, and the highest (S1) was set to 3200 AEUs. The R^2 value of the standard curves are depicted in the graph. b Stability of the standard solutions under different environmental conditions. We analyzed the stability upon storage for 96 h at 2–8 °C, room temperature (RT), 37 °C or upon 10 freeze and thaw cycles. c Stability of the standard solutions over time. The stability was analyzed upon storage for 7 months at 2–8 °C. d A newly thawed batch of the reference standard was compared with the old batch that had already been used for over 7 months. The dashed line depicts the cut-off for a positive antibody response, calculated as the mean OD value of the results of 120 negative human sera (Fig. 3a) plus four standard deviations.

Based on these results, the variability between the assays was calculated accordingly. Specificity of the assay was determined by using 120 human serum samples from 2014 without SARS-CoV or SARS-CoV-2 history. The specificity of the ELISA was calculated as 100% minus the percentage of sera reacting false positively with the ELISA. 95% confidence interval (CI) were determined using GraphPad Prism 8 software.

2.6. MERS-CoV and HKU1 S1 ELISA

These ELISAs were performed as described for SARS-CoV-2. The following antigens were used: 1 µg/ml HKU1 spike (S1) protein (Sino Biological, 40,021-V08H) or 0.5 µg/ml in PBS of MERS-CoV spike (S1) protein (Sino Biological, 40,069-V08H).

2.7. SARS-CoV-2 neutralization assay

Sera (starting at a dilution of 1:4) were serially diluted in 96-well culture plates in DMEM supplemented with 2% FCS, P/S and Q. A tissue culture infectious dose ($TCID_{50}$) of 100 units of SARS-CoV-2 (German isolate BavPat1/2020; European Virus Archive Global # 026 V-03883) was added to the serum dilutions in an equal volume of DMEM 2% FCS, P/S and Q. After incubation at 37 °C for 1 h, approximately 10,000 Vero C1008 cells (ATCC CRL-1586) were added to each well. Plates were then incubated at 37 °C with 5% CO2, and cytopathic effects (CPE) were evaluated at day 4 post infection. Neutralization was defined as complete reduction of CPE in serum dilutions compared to positive controls. Neutralization titers of three replicates were calculated as geometric means (reciprocal value). The lower detection limit of the assay is 8 and is determined by the first dilution of the respective serum including the added virus. Neutralization assays were performed in the BSL-4 laboratory of the Institute of Virology, Philipps-University Marburg, Germany.

2.8. Molecular cloning

The SARS-CoV-2 spike gene sequence (reference sequence **MN908947.3**) was divided in three parts (Fragment 1: amino acids (AA) M1 to N437; Fragment 2: AA S438 to D820; Fragment 3: AA L821 to T1273) and made available to Eurofins Genomics Germany GmbH for codon-optimization. The products were synthesized and provided. Resulting cDNAs were digested (Fragment 1: *XhoI/EcoRI*; Fragment 2: *EcoRI/BglII*; Fragment 3: *BglII/NheI*) and ligated into the pCAGGS vector cut with *XhoI* and *NheI*. Sequencing confirmed the correct sequence, the AA sequence corresponds to the spike protein reference sequence **QHD43416.1**. After the cloning of the SARS-CoV-2 spike gene the expression of the protein was verified by Immunofluorescence assay (IFA) and Western blot analysis.

2.9. Native surface staining

The analysis was performed as described by Krähling et al., 2016 (Krähling et al., 2016). Briefly, HuH7 cells were transfected with 1 µg empty vector (mock) or 1 µg pCAGGS-SARS-CoV-2 spike expressing constructs. At 24 h post transfection, cells were incubated with a human anti-SARS-CoV-2 serum or a test serum diluted 1:50 in blocking buffer at 2–8 °C. Then, cells were fixed with 4% paraformaldehyde for 20 min. Cells were treated 10 min with 100 mM glycine followed by 10 min incubation with blocking buffer. Secondary Alexa Fluor® 488 goat anti-human IgG (H + L) antibody (1400, Molecular Probes, A-11013) and DAPI (1 mg/ml, 1,2000) were used for detection. Between each step,

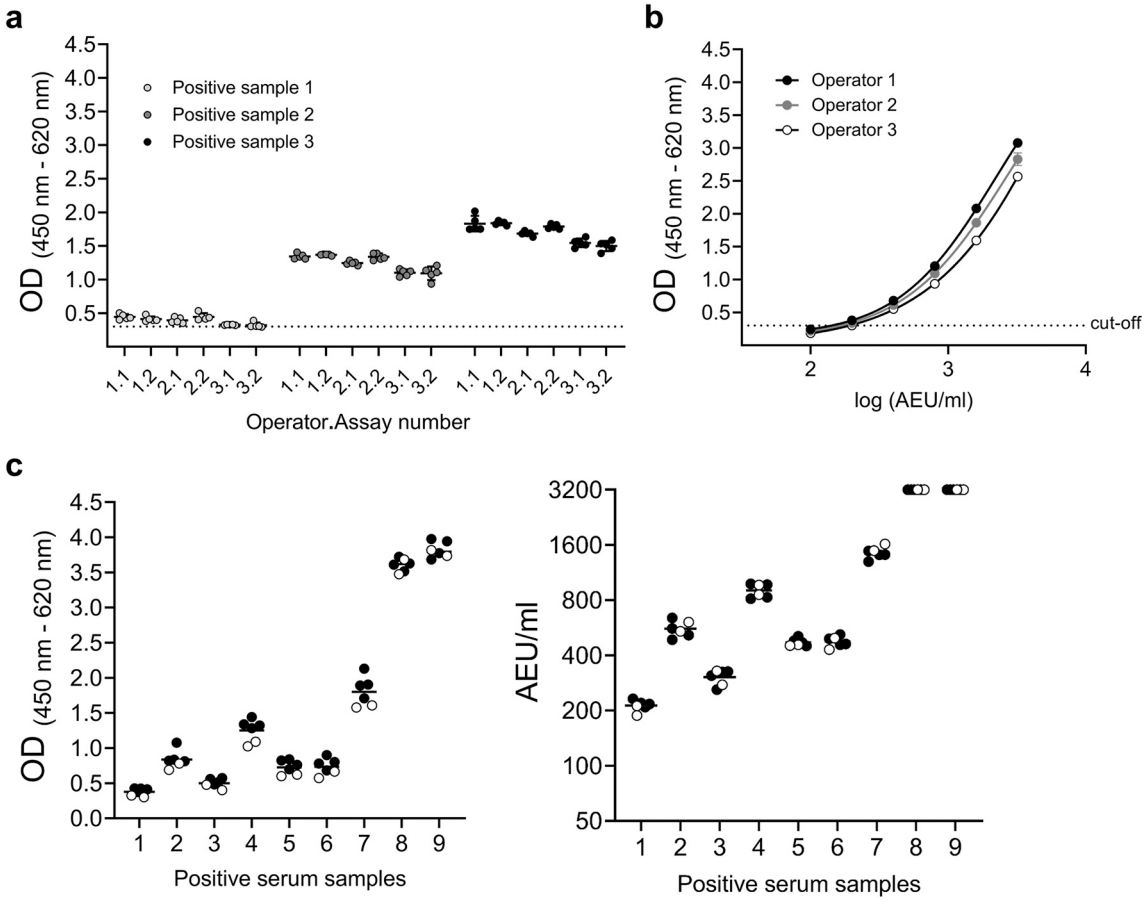

Fig. 2. Variability of the results of the SARS-CoV-2 S1 ELISA a Intra-assay variability was analyzed using three different positive serum samples in five replicates in six different assays. Three operators performed the assays. The OD values are shown. The calculated coefficients of variability (CV) are shown in Table 1. b Exemplary standard curve of each operator. Based on these curves the AEU/ml were calculated for nine different positive human serum or plasma samples. c Inter-assay variability was analyzed using nine different positive serum samples in duplicates in six different assays performed by three operators. The OD values (left graph) and the calculated AEU/ml (right graph) are shown. White circles represent the values obtained by operator three.

cells were washed three times with PBS^{++}, and all steps were performed at 2–8 °C. Cover slips were fixed with FluorSave™ Reagent (Cat-No.: 345789; Merck Millipore, USA). Microscopic analyses were performed at a magnification of 63× with a confocal laser scanning microscope (Leica SP5). All images were acquired with a laser intensity of 10% (488 nm excitation).

2.10. Western blot analysis

20 µg recombinantly expressed SARS-CoV-2 spike (S1) protein (Sino Biological, 40,591-V08H) and 15 µg SARS-CoV-2 nucleoprotein N (Sino Biological, 40,588-V08B) were separated on 10% denaturing, preparative SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were cut into small strips to allow detection of viral proteins by a small volume of human sera. Immunostaining was performed with 1:100 dilutions of human sera or plasma in PBST containing 1% (w/v) milk powder. Western blot detection was performed with HRP-conjugated secondary antibodies (1,20,000) using the Image Lab™ software and the ChemiDoc™ XRS+ Systems (BIO-RAD) for quantification.

3. Results

3.1. Development of a SARS-CoV-2 S1 ELISA

For the development of the SARS-CoV-2 S1 IgG ELISA, various parameters were optimized. Since it was not initially known whether the full-length spike protein (S1/S2) or its S1 part would provide more accurate results in serological diagnostics, both variants were compared using eleven negative sera. Using the same concentrations of the spike protein (S1/S2) or S1 showed that higher background values were obtained with S1/S2 (mean OD value of 0.278 for S1/S2 and 0.072 for S1). Therefore, S1 was chosen as the antigen. Next, the optimal coating concentration of S1 was set to 1 µg/ml (=100 ng/well) because it showed the best signal-to-noise ratio. Further, the anti-human IgG secondary antibody showed negligible binding to the coated antigen (data not shown).

To create a standard curve for the SARS-CoV-2 S1 ELISA, plasma samples of convalescent COVID-19 patients with high S1 binding activity were serially diluted and tested. The OD values obtained were used to carry out a 4-parameter logistic (4PL) regression analysis (Fig. 1a). According to the received R squared values plasma 2 was selected as reference standard. The short-term stability of the reference standard was analyzed at different temperatures and upon ten freeze and thaw cycles. The long-term stability of the reference standard was analyzed after 7 months of storage at 4 °C (Fig. 1c). The activity of the reference standard was not impaired by the different treatment and storing conditions. Only storage at 37 °C for 4 days resulted in a slight decrease in reactivity (Fig. 1b). Furthermore, a new and the old batch of the reference standard was compared and did not show significant differences (Fig. 1d). In all subsequent experiments, 4PL regression analysis of the standard curve was used to calculate arbitrary ELISA units (AEUs) for positive samples. The standard with the lowest antibody concentration (S6) was set to 100 AEUs/ml, and the highest concentration (S1) was set to 3200 AEU/ml.

Table 1
Intra- and inter-assay variability.

	Coefficient of variability (CV in %)		
	OD value (450 nm – 620 nm)		AEU/ml
	Intra-assay	Inter-assay	
Positive sample 1 (low; $n = 4$)	8.8%	14.1%	7.0%
Positive sample 2 (medium; $n = 3$)	3.8%	13.3%	8.7%
Positive sample 3 (high; $n = 2$)	3.3%	2.8%	NA[a]
Mean CV (%)	5.3%	10.1%	7.9%

The intra-assay coefficient of variability (CV) was determined by calculating the standard deviation of the means/the means of 5 replicates for each serum sample (Fig. 2a). Inter-assay variability was calculated using the mean OD values of 9 different samples (two highly positive, three medium and four low positive samples; Fig. 2c) or the interpolated value for each sample obtained by 4PL regression analysis (AEU/ml). The mean of six independent assays performed by three operators is shown.

[a] Samples with results above the standard with the highest concentration of antibodies will be assigned >3200 AEU/ml, resulting in no variability.

3.2. Key performance indicators of the SARS-CoV-2 S1 ELISA

To determine the intra-assay variability of the newly developed SARS-CoV-2 S1 ELISA, three different positive samples from convalescent COVID-19 patients were tested in 5 replicates by different operators on different days (Fig. 2a). The calculated intra-assay variability of the results was 5.3% (Table 1). The inter-assay variability determined by testing nine samples on three different days by three independent operators (a total of 6 assays) was 10.1% (OD values) or 7.9% (AEU/ml) (Table 1).

Interpolation of AEU/ml (Fig. 2b) according to the standard curve on each plate (three examples are shown in Fig. 2b) revealed a lower inter-assay variability for low and medium reactive samples (Table 1, Fig. 2c). The overall variability of the results is reduced depending on the operator (compare the results of operator 3, represented by the white circles, Fig. 2c).

The cut-off value of the ELISA was determined by analyzing the OD values of 120 negative sera from 2014 with no history of SARS-CoV infection (Fig. 3a). The cut-off value was calculated as the average of the OD values of these negative sera plus 4 standard deviations and was accordingly set to 0.3. According to this cut-off, one (0.8%) of the 120 negative samples reacted false-positively with the SARS-CoV-2 S1 antigen in the ELISA at 240 AEU/ml. This serum was neither lipemic nor hemolytic, but it was the only serum among the 120 tested that was positive for anti-HBe IgGs. The analysis of this serum sample by Western blot (Fig. 3b) and native immunofluorescence (nIFA, Fig. 3c) did not confirm the presence of anti-SARS-CoV-2 spike protein-specific antibodies. In both assays a human COVID-19 convalescent serum was used as positive control. Fourteen of the 120 tested serum samples were

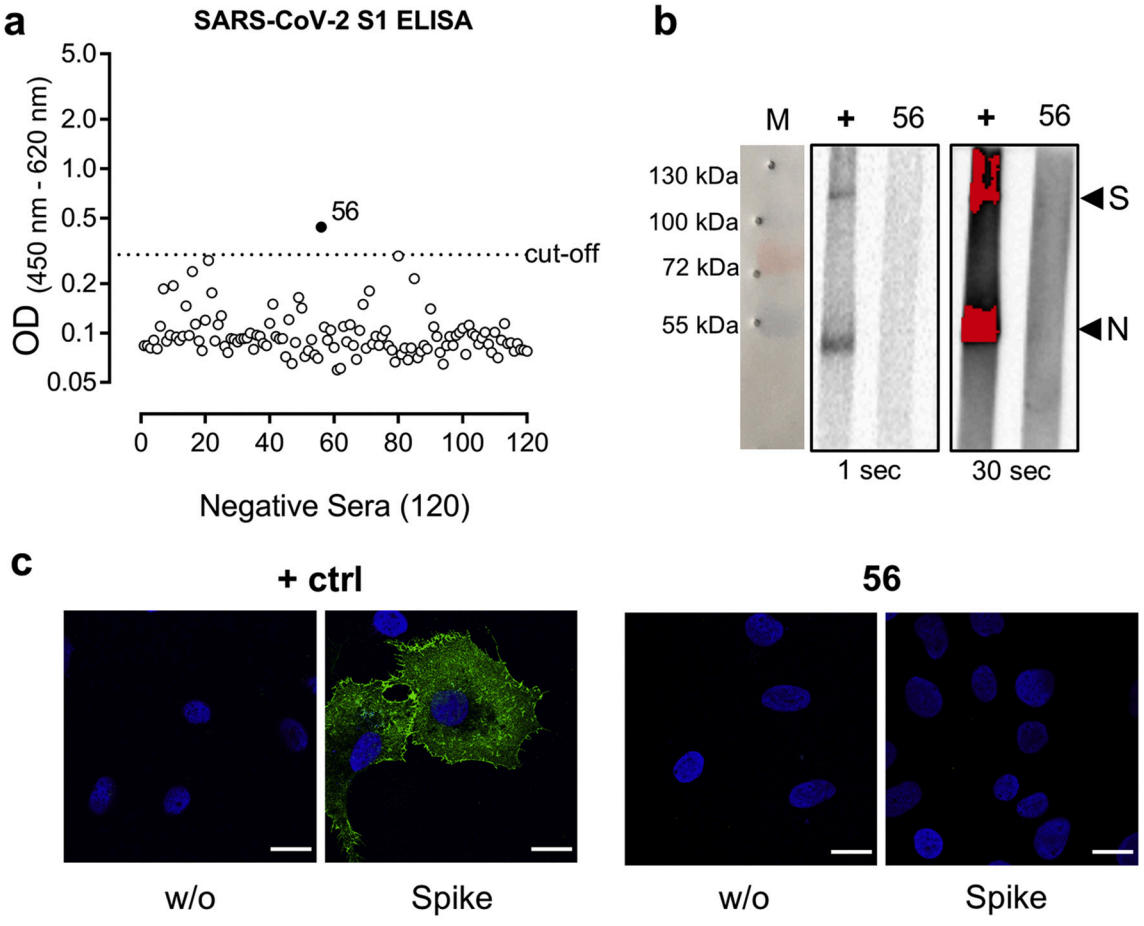

Fig. 3. Specificity of the SARS-CoV-2 S1 ELISA a 120 randomly selected anonymous human serum samples without SARS-CoV or SARS-CoV-2 history were analyzed by SARS-CoV-2 S1 ELISA (dilution 1:101). The cut-off of the assay (OD = 0.3) was set to the mean value ($OD_{mean} = 0.104$) of the sera depicted here plus 4 standard deviations (SD = 0.049). b Serum sample #56 was analyzed for the detection of SARS-CoV-2 spike (S) and nucleoprotein (N) by Western blot (dilution 1:100). A low-sensitivity HRP substrate was used to detect the enzymatic activities of HRP-coupled secondary antibodies. The exposure times of 1 s and 30 s are displayed. The positive control was obtained from a COVID-19 patient (dilution 1:100, positive control, +). c Native Immunofluorescence analysis was performed to confirm the results. Microscopic analyses were performed at a magnification of 63× with a confocal laser scanning microscope (Leica SP5). All images were acquired with a laser intensity of 10% (488 nm excitation). Scale bar: 20 μM.

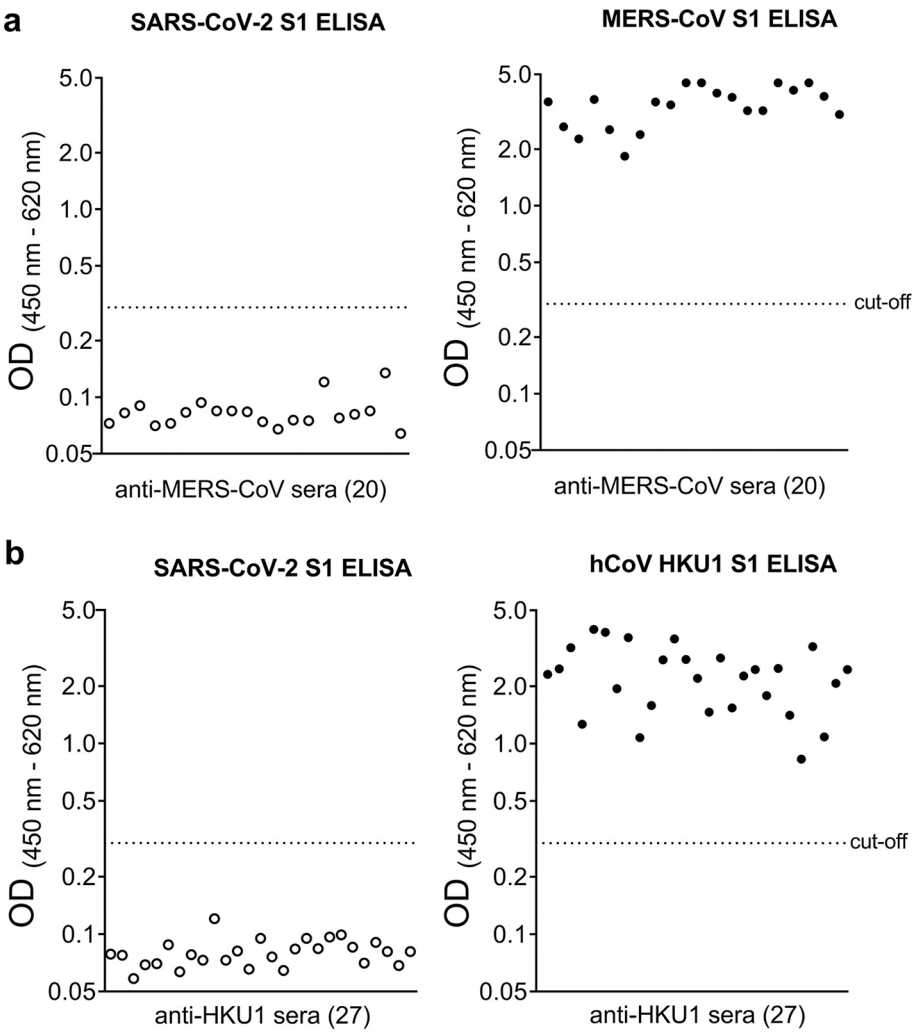

Fig. 4. Cross-reactivity of antibodies (IgG) detecting other hCoV a 20 sera with MERS-CoV S1-specific or b 27 sera with HKU1 S1-specific antibodies were analyzed in the SARS-CoV-2 S1 ELISA and in the respective other hCoV ELISA.

lipemic, and four were hemolytic. Based on these results, the specificity of the ELISA was calculated to be 99.2% (95% CI, 95.4% - 99.96%). The present ELISA was also used to detect SARS-CoV-2 specific antibodies within 1000 serum samples from healthy human volunteers. The results obtained showed that 5 of these serum samples were positive, 24 of these serum samples were false positive (unpublished results). Thus, the SARS-CoV-2 S1 ELISA shows an overall specificity of 97.8% (95% CI, 96.7% - 98.5%) corresponding to 25 false positive results from 1115 negative samples tested.

To further assess the specificity of the SARS-CoV-2 S1 ELISA the cross-reactivity of sera containing anti-MERS-CoV or anti-HKU1 IgG antibodies was analyzed. To this end, twenty human serum samples of an open-label, phase 1 trial to assess safety and immunogenicity of a modified vaccinia virus Ankara vector vaccine candidate for Middle East respiratory syndrome (Koch et al., 2020) (ClinicalTrials.gov, NCT03615911, and EudraCT, 2014–003195-23) were used. It was shown that IgG antibodies of these sera bound to the MERS-CoV S1 protein, but not to the SARS-CoV-2 S1 protein (Fig. 4a). To analyze the cross-reactivity of antibodies against other human CoVs, we used 27 serum samples that were screened for reactivity to the HKU1 S1 antigen. Those sera were also negative in our SARS-CoV-2 S1 ELISA (Fig. 4b).

3.3. Detection of SARS-CoV-2 S1 protein-specific antibodies

To determine whether the SARS-CoV-2 S1 ELISA was able to monitor the development of antibodies in COVID-19 patients, serial serum or plasma samples from twenty-three COVID-19 patients were analyzed between day 2 before (-2) until day 69 after the patient's first positive SARS-CoV-2 RT-PCR result (Table 2). Unfortunately, precise and reliable information on the onset of the disease of all patients are not available.

In this experiment, the OD values are displayed instead of the AEU/ml to show the results of all samples, including those that were below the cut-off of the SARS-CoV-2 S1 ELISA (Fig. 5a). Overall, the antibody titer profiles of the twenty-three COVID-19 patients were highly variable (Fig. 5a). Patients that were negative or weakly positive in the ELISA at the first time point of sampling (patient numbers: 2, 3, 5, 7, 9, 10, 11, 15, 16, 17, 21, 22) developed anti-SARS-CoV-2 IgG antibodies between day -1 and day 36 after they were tested positive for SARS-CoV-2 by RT-PCR. Ten of the COVID-19 patients (patient numbers: 1, 4, 6, 12, 13, 14, 18, 19, 20, 23) became ELISA-positive at the first time point of sampling at days 0 to 19 relative to the positive result in RT-PCR. The date of the first positive RT-PCR is not known for patient #8.

To confirm that the results were reliable, neutralization tests with authentic SARS-CoV-2 were performed. Neutralizing antibodies were detected in all samples that were positive in the SARS-CoV-2 S1 ELISA (Fig. 5b). In fact, it was found that three early samples that were still negative in the S1-specific IgG ELISA already contained small amounts of neutralizing antibodies (patient number: 7 (1st sample), 11 (2nd sample) and 21 (1st sample)). Three of the patients (#10, #11, #15) developed only low titers of S1-specific antibodies that were reactive in

Table 2
COVID-19 patient information: dates of sampling.

COVID-19 patient	Positive RT-PCR	Serum/plasma samples					
		First		Second		Third	
		date	Days post PCR	Date	Days post PCR	date	Days post PCR
1	31.03.2020	31.03.2020	0	08.04.2020	8	NA	NA
2	29.03.2020	31.03.2020	2	07.04.2020	9	14.04.2020	16
3	03.04.2020	01.04.2020	-2	14.04.2020	11	NA	NA
4	02.04.2020	02.04.2020	0	15.04.2020	13	NA	NA
5	27.03.2020	27.03.2020	0	06.04.2020	10	10.04.2020	14
6	21.03.2020	24.03.2020	3	27.03.2020	6	31.03.2020	10
7	01.04.2020	01.04.2020	0	07.04.2020	6	15.04.2020	14
8[a]	NA	27.03.2020	NA	03.04.2020	NA	08.04.2020	NA
9	02.04.2020	01.04.2020	-1	07.04.2020	5	14.04.2020	12
10	27.02.2020	27.02.2020	0	12.03.2020	14	NA	NA
11	03.02.2020	12.02.2020	9	19.02.2020	16	10.03.2020	36
12	28.01.2020	12.02.2020	15	10.03.2020	42	06.04.2020	69
13	05.02.2020	19.02.2020	14	10.03.2020	34	06.04.2020	61
14	31.01.2020	19.02.2020	19	10.03.2020	39	06.04.2020	66
15	03.02.2020	11.02.2020	8	10.03.2020	36	06.04.2020	63
16	10.11.2020	13.11.2020	3	23.11.2020	13	01.12.2020	21
17	20.11.2020	20.11.2020	0	24.11.2020	4	30.11.2020	10
18	04.11.2020	16.11.2020	12	27.11.2020	23	29.11.2020	25
19	12.11.2020	24.11.2020	12	30.11.2020	18	NA	NA
20	30.11.2020	30.11.2020	0	01.12.2020	1	NA	NA
21	25.11.2020	25.11.2020	0	29.11.2020	4	NA	NA
22	12.10.2020	11.10.2020	-1	24.11.2020	12	NA	NA
23	23.10.2020	02.11.2020	10	09.11.2020	17	NA	NA

For patients #11 to #15 data are taken from Kreer et al. (2020). Date of diagnosis corresponds to the first positive RT-PCR.
[a] The date of the first positive RT-PCR result is not known. The patient was transferred from another hospital, where first RT-PCR positive result was obtained.

the ELISA. Nevertheless, their sera neutralized SARS-CoV-2, although with lower titers compared to the other patients.

To further validate the newly developed ELISA, all sera were re-analyzed using the commercially available IgG- (Fig. 5c) and IgA- (Fig. 5d) specific SARS-CoV-2 ELISAs (EUROIMMUN). Overall, the antibody titer profiles were comparable. The three early samples that were negative in the newly developed SARS-CoV-2 S1 ELISA (patient number: 7 (1st sample),11 (2nd sample) and 21 (1st sample)) were also negative in the IgG-specific commercial S1 ELISA. Two of them (patient number: 7 (1st sample),11 (2nd sample)) were tested positive using the IgA-specific ELISA. Using both IgG-specific ELISAs, the results of some early samples and samples from those who developed only low titers varied in six of the COVID-19 cases. The first sample of patients #2, #5, #9, the third sample of patient #11,the first and the third sample of patient #15 and the first sample of patient #17 were tested negative using the commercially available IgG-specific ELISA but were tested positive using the newly developed ELISA. Importantly, the neutralization test confirmed the positivity of these samples. Of those seven sera four were tested positive using the IgA-specific ELISA (patient: #5, #9 and #17 (1st sample) and #11 (3rd sample)). Overall, the testing of 59 samples from twenty-three COVID-19 patients in the different ELISAs gave the following results: The newly developed SARS-CoV-2 S1 ELISA detected S1 protein-specific antibodies in 52 of 59 samples, whereas the IgG- and IgA-specific ELISAs from EUROIMMUN detected antibodies in 45 or 51 samples, respectively. The neutralization test showed that 55 out of 59 samples were indeed positive for SARS-CoV-2 S-specific antibodies. In addition, single serum samples obtained from 29 further COVID-19 patients were analyzed in the SARS-CoV-2 S1 ELISA and the neutralization test. The results of all tested serum samples ($n = 88$) are summarized in Fig. 6a. Twelve of these samples were tested negative in the in-house ELISA (Fig. 6a) and twenty-four were tested negative in the EUROIMMUN IgG ELISA (Fig. 6b). Four or twelve of these samples were positive for neutralizing antibodies (Fig. 6a and b, red circles). The correlation analysis showed a good correlation between the different IgG-specific ELISAs (Fig. 6c) and between the neutralization test and the in-house ELISA (Fig. 6d).

4. Discussion

In the present study, we developed an IgG ELISA detecting SARS-CoV-2 spike protein (S1 domain)-specific antibodies. The decision to use S1 instead of the full-length spike protein (S1/S2) was made based on the comparison of the background signal of negative sera on the two proteins that was more favorable for S1. This result is in line with the observation that S2 is more conserved in different coronaviruses compared to S1, explaining the background activities of SARS-CoV-2-negative sera (Okba et al., 2020). The precision of the SARS-CoV-2 S1 ELISA was determined by measuring the intra-assay variation (CV) for three positive samples and the inter-assay CV for nine positive samples. The results (Table 1) showed a high reproducibility and comply with the generally accepted standards of intra-assay and inter-assay variabilities of no more than 15 and 20%, respectively (Jacobson, 1998). A set of standards was used to account for plate-to-plate and inter-operator variability. The standards were stable upon short term storage (4 days) at different temperatures, the impact of long-term storage will be continuously determined.

The specificity of the SARS-CoV-2 S1 ELISA was measured by analyzing 120 negative human serum samples - one of them was tested false positive. In addition, the SARS-CoV-2 S1 ELISA, described here, was used to analyze 1000 serum samples from volunteers for SARS-CoV-2 spike-specific antibodies (unpublished results). Of these, 5 serum samples were truly positive and 24 serum samples gave false positive results. Based on these results, the specificity was calculated to be 97.8% (95% CI, 96.7% - 98.5%) corresponding to 25 false positive samples of 1115 negative serum samples tested. The binding of antibodies specific for other coronaviruses such as HKU1- and MERS-CoV to the spike protein of SARS-CoV-2 showed no cross reactivity with SARS-CoV-2 S1. However, due to the limited number of serum samples and the lack of serum samples that react to other human coronaviruses such as NL63 or OC43, the low cross-reactivity must be considered with caution.

The sensitivity of the assay was tested by analyzing multiple samples from twenty-three COVID-19 patients (Fig. 5) and individual samples from 29 COVID-19 patients (in total $n = 88$). Seroconversion was found

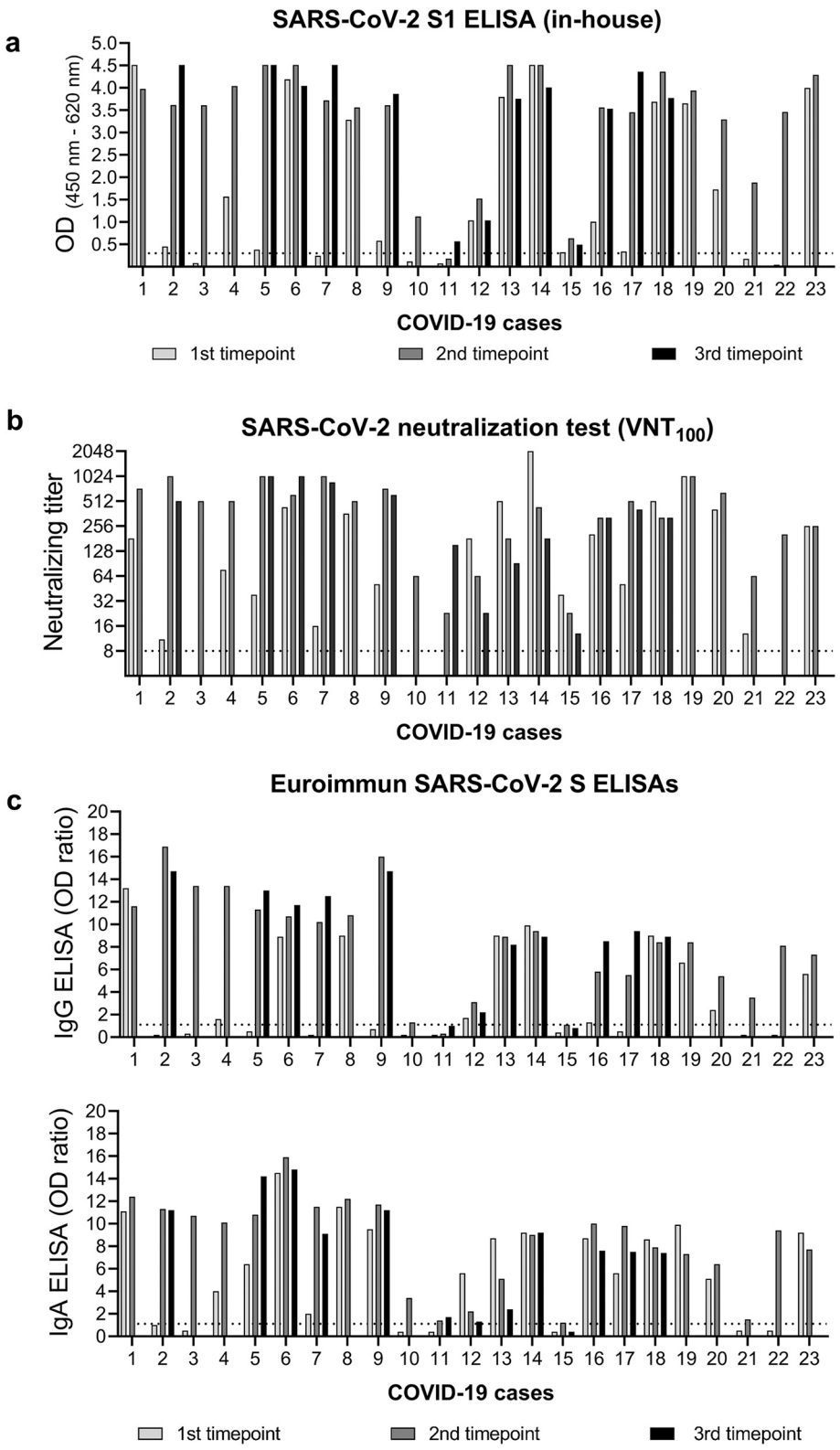

Fig. 5. Detection of SARS-CoV-2 S1 protein-specific antibodies in COVID-19 patients a Consecutive serum or plasma samples from 23 COVID-19 patients (dilution 1:101) were analyzed using the SARS-CoV-2 S1 ELISA. The sample OD values are displayed to visualize the results of all samples. b Neutralization of authentic SARS-CoV-2 by the respective samples. c The same samples were analyzed using the commercially available SARS-CoV-2 S-specific IgG (upper panel) and IgA (lower panel) ELISAs.

in all COVID-19 patients in the ELISA and in the neutralization test. From 12 negative samples in the SARS-CoV-2 in-house ELISA four were tested positive in the neutralization test. Based on the results, the sensitivity of the SARS-CoV-2 S1 ELISA was calculated to be 95.0% (95% CI: 98.0% - 87.8%) corresponding to 76 out of 80 positive samples detected. As the sample size is small, the sensitivity of the assay will continue to be analyzed as more samples become available from COVID-19 patients. In the commercially available IgG-specific ELISA 14 sera were tested negative or borderline that were shown to be positive in the neutralization test. Based on the results, the sensitivity was calculated to be 82.5% (95% CI: 89.3% −72.7%) corresponding to 66 out of 80 positive samples detected. This result is in line with previously published data showing a sensitivity of this ELISA of 71.1% corresponding to 32 out of 45 samples (Kohmer et al., 2020a). But, also higher sensitivity

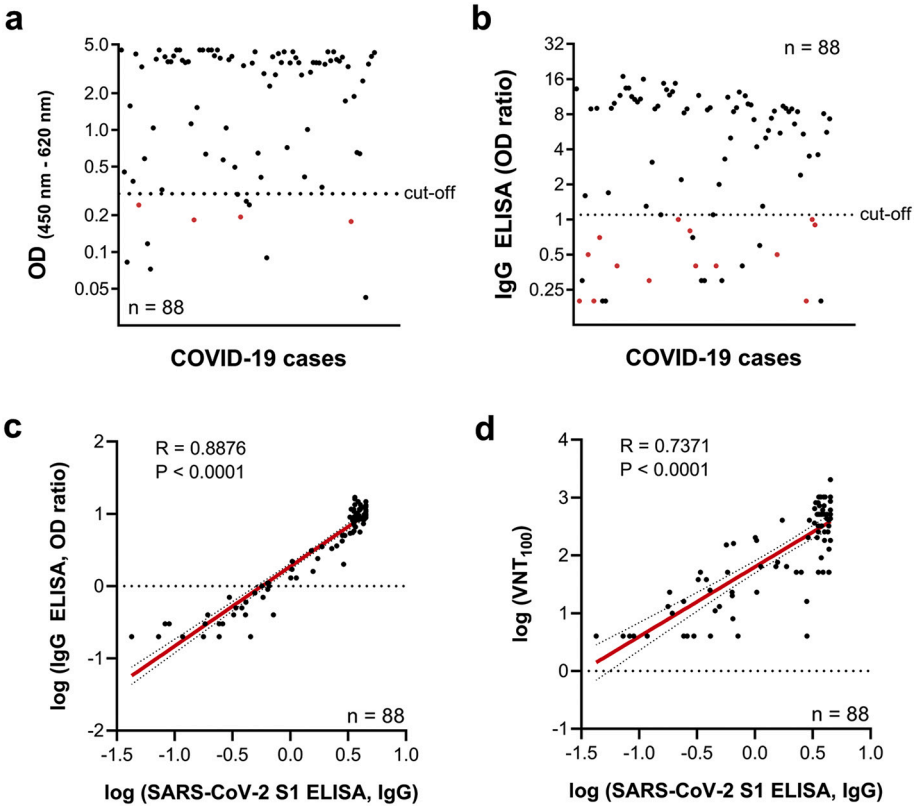

Fig. 6. Detection of SARS-CoV-2 S1 protein-specific antibodies and correlation analyses a - b Serum or plasma samples of 52 COVID-19 patients (n = 88, dilution 1:101) were analyzed using the SARS-CoV-2 S1 in-house or EUROIMMUN IgG ELISA. Samples that were tested negative in the ELISA and positive in the neutralization test are represented by red dots. c Correlation of the in-house SARS-CoV-2 S1 ELISA and the EUROIMMUN (EI) IgG ELISA. d Correlation of binding antibodies detected by the in-house SARS-CoV-2 ELISA with live virus neutralizing titers (VNT100). Red lines reflect the best linear fit relationship between these variables, dotted lines visualize 95% confidence intervals. P and R values reflect two-tailed Spearman rank-correlation tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rates (92.86%; 100 samples tested) and a good correlation with a neutralization test were found for this commercial ELISA (Weidner et al., 2020). Correlation analysis of the SARS-CoV-2 S1 in-house ELISA with the EUROIMMUN IgG ELISA and the virus neutralization test using authentic SARS-CoV-2 also revealed a good correlation (Fig. 6). Analyses by others (Meyer et al., 2020) showed that the diagnostic accuracy of the commercially available IgG SARS-CoV-2-S1 ELISA (EUROIMMUN), was almost optimal for a sample collective of 181 laboratory-confirmed COVID-19 cases and 326 controls. Because the COVID-19 patient's antibody titer profiles were different, including high and very low levels of antibodies, and neutralizing antibodies were found in four samples that were still negative in the SARS-CoV-2 S1 in-house ELISA, it may be important that samples are tested every two weeks using an ELISA if it is very likely that an infection has already gone through. In addition, analysis of other classes of antibodies (IgA) can be helpful to detect seroconversion earlier, as it was shown that IgA significantly contribute to early neutralization of SARS-CoV-2 (Sterlin et al., 2020). We recommend performing confirmation tests such as neutralization tests or native immunofluorescence analyzes to avoid false positive results. Since this is generally not feasible for routine diagnostics, the analysis of consecutive samples is the means of choice here. Successive samples from vaccinated individuals can be reliably analyzed because the background reactivity of the serum from each individual is determined before vaccination.

5. Conclusion

In summary, we have developed an indirect ELISA to detect IgG antibodies directed against the S1 subunit of the SARS-CoV-2 spike protein in the sera of COVID-19 patients. The results underscore the high specificity, sensitivity, and precision of the SARS-CoV-2 ELISA, which is suitable for epidemiological studies and for assessing the immunogenicity of current vaccine candidates against SARS-CoV-2 as the spike protein is used by most vaccines as target protein.

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Ethics approval

This study was carried out in accordance with the Declaration of Helsinki and the guidelines of the International Conference for Harmonization for Good Clinical Practice. Informed consents to use serum and plasma for scientific research were obtained.

Declaration of Competing Interest

None.

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