Analytical characterization of the SARS-CoV-2 EURM-017 reference material

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ABSTRACT

Background: Current serological methods for SARS-CoV-2 lack adequate standardization to a universal standard reference material. Standardization will allow comparison of results across various lab-developed and commercial assays and publications. SARS-CoV-2 EURM-017 is human sera reference material containing antibodies directed against SARS-CoV-2 proteins, S1/S2 (full-length spike [S]), S1 receptor-binding domain (S1 RBD), S1, S2, and nucleocapsid (N) protein. The goal of this study was to characterize five antigen-specific serum fractions in EURM-017 for standardization of serology assays.
Methods: Five antigen-specific serum fractions were affinity purified, quantified, and $PRNT_{50}$ titers compared. Standardization methods were established for two anti-S1 RBD (IgG and Total Ig) and one N protein assay. For the anti-S1 RBD assays, standardization involved determining assay index values for serial dilutions of S1-RBD anti-sera. Index values for the anti-S1 RBD IgG assay and $PRNT_{50}$ titers were determined for 44 symptomatic COVID-19 patient sera. The index values were converted to EURM-017 ug/mL.
Results: Anti-sera protein content was as follows: S1 (17.7 μg/mL), S1 RBD (17.4 μg/mL), S1/S2 (full-length S) (34.1 μg/mL), S2 (29.7 μg/mL), and N protein (72.5 μg/mL). S1 anti-serum had the highest neutralization activity. A standardization method for S1 RBD anti-serum and an anti-S1 RBD IgG assay yielded the linear equation ($y = 0.75x-0.10$; y = index, x=μg/mL anti-serum). Patient sample index values for the S1-RBD IgG assay correlated well with $PRNT_{50}$ titers (Pearson r = 0.84). Using the equation above, patient index values were converted to standardized μg/mL.
Conclusions: Standardization of different lab-developed and commercial assays to EURM-017 antigen-specific anti-sera will allow comparison of results across studies globally due to traceability to a single standard reference material.

1. Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is a highly infectious virus that emerged in Wuhan, China in late 2019 [1]. Since then, SARS-CoV-2 has spread rapidly throughout the world causing the devastating Coronavirus Disease-2019 (COVID-19). COVID-19 has crippled daily life and economies, and in March 2020, COVID-19 was declared a pandemic [1,2]. Antibodies appear approximately one to three weeks post symptom onset in most patients and are produced in both symptomatic and asymptomatic infection [3]. A variety of commercial and "in-house" lab-developed immunoassays detect antibodies

(IgM, IgG, and IgA) to SARS-CoV-2 proteins, mainly those related to the immunodominant spike (S) protein and nucleocapsid (N) protein [3,4]. The S protein is a 1273 amino acid (aa) long transmembrane glycoprotein that harbors two domains, S1 (aa 14-685) and S2 (aa 686-1273) [3]. S1 mediates recognition and binding of the viral receptor (ACE2) on host cells, and S2 facilitates viral fusion and entry [5,6]. The S1 domain contains an N-terminal domain (aa 14-305) and the receptor-binding domain (S1 RBD, aa 319-541) that directly binds ACE2 [7]. Antibodies to S1 RBD have been shown to account for about 90% of the neutralizing activity in patient sera [8]; although additional neutralizing activity targets non-S1 RBD sites on S protein such as the N-terminal

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domain and S2 fusion peptide (aa 788-806) regions [9–12]. Multiple studies using patient sera have shown correlations between various anti-S and anti-S1 RBD IgG assays and neutralizing antibody titers [7,11,13–18]. In addition, correlations have been found between disease severity and various anti-S and anti-S1 RBD IgG assays, and between disease severity and neutralizing antibody titers [11,17,18]. Multiple vaccines that are available or in development target or include the S1 RBD, and antibodies to this region in vaccinated serum have demonstrated neutralizing activity [19–25]. Some studies have suggested that anti-N protein antibody assay values have correlated with neutralization [17,26], but to a lesser extent than anti-S1-related antibody assays [17].

Despite unprecedented advances in our understanding of COVID-19 and in providing effective vaccines, several questions remain. These include a better understanding of the immune correlates of protection in infected, re-infected, vaccinated individuals, donor convalescent plasma, and the length of time that immunity persists. For the various studies reported, including vaccine studies, antibody levels in patient sera were determined using various lab-developed and commercial assays and cutoffs. This limits the ability of researchers to confidently compare results across studies. Standardization of assays is a way to allow comparison of results when using the different assays around the world and can be accomplished with reference materials that are well characterized. Recently, the United States (U.S.) Centers for Disease Control and Prevention (CDC) (Atlanta, GA, U.S.) highlighted the need for standardized SARS-CoV-2 quantitative immunoglobulin (IgG) and neutralization assays [27]. EURM-017 is new reference material that has been made available for standardization of a range of lab-developed and commercial immunoassays and neutralization assays, globally. It should be noted that standardization is not for individual patient management.

The goal of this study was to describe the purification and analytical characterization of five antigen-specific serum fractions (S1 RBD, N protein, S1, S2, and the S1/S2 [full-length S]) present in EURM-017, for standardization of anti-SARS-CoV-2 serology assays.

2. Materials and methods

2.1. Characterization of SARS-CoV-2 EURM-017 reference material (human serum with antibodies against SARS-CoV-2)

EURM-017 is quality control sera for immunoassay-based in vitro diagnostic devices (International Federation of Clinical Chemistry [IFCC] and the European Commission, Joint Research Centre Directorate F – Health, Consumers and Reference Materials Retieseweg 111B-2440 Geel, Belgium [28]). It consists of a pool of serum samples obtained from different plasma donors who had a SARS-CoV-2 infection 10 to 16 weeks prior to the plasma collection. Five anti-SARS-CoV-2 antibody serum fractions (S1 RBD, N protein, S1, S2, and the S1/S2 [full-length S]) in EURM-017 were affinity purified using biotinylated SARS-CoV-2 antigens coupled to streptavidin-coated latex particles.

2.1.1. SARS-CoV-2 biotin conjugate preparation

Fifty milligrams (50 mg) of each of the five viral proteins was conjugated to a 10-fold molar excess of NHC-LC-Biotin for 1 h. The unreacted NHS-LC-Biotin was removed from the conjugated protein by gel filtration (G25).

2.1.2. Magnetic particle preparation

Three (3) grams of streptavidin-coated microparticles (Invitrogen dynabeads M−280, Thermo Fisher Scientific) was washed twice with 50 mL of 0.01 M PBS, pH 7.4 + 0.1% TWEEN 20 solution (Buffer A). The microparticles were centrifuged (935 × g, 10 min) after each wash. After the final wash, the microparticles were reconstituted to a final particle concentration of 50 mg/mL.

2.1.3. S1 RBD, N protein, S1, S2, and S1/S2 (full-length S) affinity support

Ten milliliters (10 mL) of the 50 mg/mL washed microparticles was incubated with 2.5 mg of the SARS-CoV-2 antigen protein biotin conjugate (final concentration = 5 μg of protein conjugate/mg microparticle) at room temperature, 1 h, after which the particles were washed with 50 mL Buffer A three times. After each wash, the particles were centrifuged (935 × g, 10 min). The final concentration of microparticles was 50 μg/mL (10 mL final volume) in Buffer A.

2.1.4. Purification of five antibody fractions in EURM-017

Buffer A was removed from microparticles by centrifugation (935 × g, 10 min). EURM-017 (10 mL) was added to and incubated with the microparticles at room temperature, 1 h with rocking. The microparticle suspension was centrifuged (935 × g, 10 min) and washed several times, with 50 mL of Buffer A for each wash, until the Optical Density at 280 nm (OD_{280}) was < 0.05. After removing washing solution, antibodies were eluted with 50 mM citrate buffer containing 0.1% TWEEN 20, pH of 3.00 (1.5 mL) at room temperature, 15 min. The solution was centrifuged, the eluant transferred to another tube and neutralized using 1 M Tris buffer + 0.1% TWEEN 20 solution, pH 11.00 (500 μL). The purified serum fractions were dialyzed using Buffer A solution and then concentrated to approximately 1.0 mL using 30 K NMWL centrifugal tubes. Finally, the protein concentration was determined by measuring the OD_{280}.

2.2. Plaque reduction neutralization assay (PRNT)

PRNT assays were performed using the above purified serum fractions on Vero E6 cells and the icSARS-CoV-2 (USA-WA1/2020 strain) (Centers for Disease Control and Prevention and World Reference Center of Emerging Viruses and Arboviruses). Vero E6 cells (7×10^5 cells) were cultured in 6-well plates. The following day, two-fold serial dilutions (1:25 to 1:800) of each anti-serum sample and anti-serum-free control samples (125 μL; n = 6) were combined with virus (125 μL) at 800 plaque-forming units (pfu)/mL (100 pfu/125 μL) to achieve final dilutions of 1:50 to 1:1600. The mixtures were incubated at 37 °C, 1 h. Culture medium in Vero E6 plates was then replaced with sample/virus or sample-free/virus solution (200 μL), starting with the least diluted sample/virus sample (1:50). The plates were incubated with rocking at 37 °C, 45 min, after which a mixture of 1.6% agarose and 2x media (2 mL) was quickly added to each well. The plates remained undisturbed for the agarose to solidify, 5 to 10 min, and incubated at 37 °C, 2 to 3 days, until plaques were visible. Neutral Red stain was added for 2 to 3 h and plaques in each well were counted. The percent neutralization for each dilution was calculated according to the formula:

$$\%neutralization = (1 - [\# \, plaques \, with \, Ab / \# \, plaques \, with \, no \, Ab]) \times 100$$

Control plates were infected with each batch and used to determine non neutralized values for percentage calculation. The average of the used wells was included in the raw data.

2.3. Serology assays and platforms

Performance was determined for the Atellica® IM SARS-CoV-2 IgG (sCOVG) Assay, Atellica® IM SARS-CoV-2 Total (COV2T) Assay (Siemens Healthineers, Tarrytown, NY, U.S.), and cobas (ELECSYS Anti-SARS-CoV-2 assay) (Roche Diagnostics, Indianapolis, IN, U.S.) [29–31]. Assays were used as defined in the individual instructions for use. The sCOVG and COV2T assays are semi-quantitative assays that can be used to determine antibody levels and neutralization titers. At the time of the study, all assays were authorized under the U.S. Food and Drug Administration Emergency Use Authorization (EUA). Table 1 summarizes the properties of each assay.

2.4. Specificity and standardization of anti-S1 RBD and anti-N protein serology assays

Each of the five anti-sera was adjusted to a concentration of 10 μg/

Table 1
Summary characteristics of assays.

Manufacturer	Assay	Platform	Principle of test	Antigen	Ig	Reactive positive cutoff	Measuring range
Siemens Healthineers	SARS-CoV-2 IgG (sCOVG)	Atellica IM Analyzer	2-step automated sandwich chemiluminescent immunoassay (Capture:Antigen coated microparticles. Detection: Mouse monoclonal anti-human IgG Ab labeled with acridinium ester)	S1 RBD	IgG Qualitative and semi-quantitative; quantitative correlation of Atellica IM sCOVG U/mL (Index values) versus PRNT$_{50}$	≥1 Index	0.50–150 Index
Siemens Healthineers	SARS-CoV-2 Total (COV2T)	Atellica IM Analyzer	1-step automated sandwich chemiluminescent immunoassay (Capture: Antigen coated microparticles. Detection: Recombinant S1 RBD antigen labeled with acridinium ester)	S1 RBD	Total Ig (IgG and IgM)	≥1 Index	0.50–75.00 Index
Roche	ELECSYS Anti-SARS-CoV-2	cobas	Sandwich chemiluminescent immunoassay (biotinylated recombinant antigen + recombinant antigen labelled with ruthenium complex)	N protein	Total Ig (IgG, IgM)	>1	

mL and two-fold serially diluted to 0.31 μg/mL. Then, each diluted sample was tested with two anti-S1 RBD assays to obtain index values. For the anti-N protein assay, the N protein-specific anti-serum was adjusted to 100 μg/mL and two-fold serially diluted to 0.78 μg/mL.

2.5. Patient sera PRNT$_{50}$ correlation to serology index values

Here, we evaluated the extent to which the sCOVG test results reflected the presence in serum of antibodies with ability to neutralize SARS-CoV-2. Serum samples were collected from 44 symptomatic outpatients with RT-PCR and serology (Siemens Healthineers assays, SARS-CoV-2 IgG (sCOVG) and SARS-CoV-2 Total [COV2T])-confirmed SARS-CoV-2 infection. Siemens Healthineers provided 44 samples to UTMB for testing. Eleven (11) serum samples were from Serologix (New Hope, PA, www.serologix.com, US); 17 samples were from Antibody Systems (Hurst, TX, US, www.antibodysystems.com); and 15 samples were from New York Biologics (Southampton, NY, US, www.newyorkbiologics.com); one sample of EURM-017 was also included. Serum samples were diluted and processed as above for EURM-017 reference material. Index values were converted to EURM-017 values in μg/mL using the equation y = 0.75x −0.10. This study was retrospective, for which the human samples collected had relevant Institutional Review Board approvals and patient consent.

2.6. Statistics

PRNT$_{50}$ values were calculated using PRISM 7.0 software for non-linear regression: Sigmoidal, 4PL, X is log(concentration); constrains on the top of 1 and bottom of 0.

3. Results

3.1. Characterization of EURM-017 human serum reference material

Five antigen-specific anti-SARS-CoV-2 polyclonal antibody fractions ((S1 RBD, N protein, S1, S2, and the S1/S2 [full-length S]) were purified from EURM-017 human serum. The antibody yield from each of the five purifications is shown in Table 2. The concentration was highest for antibodies directed against N protein (by 2 to 3-fold), followed by antibodies directed against S1/S2 (full-length S), S2, S1, and S1-RBD.

The anti-serum concentration at each dilution prepared for each of the five purifications used for PRNT is shown in Table 3.

3.2. PRNT at different dilutions and PRNT$_{50}$ estimates

The percent plaque reduction at each dilution, the dilution, and amount required for PRNT$_{50}$ and PRNT$_{90}$ for each of the five purifications are shown in Table 4. The amount required for PRNT$_{50}$ and PRNT$_{90}$

Table 2
The protein yield from each of the five affinity purified serum fractions.

Anti-sera specificity	Volume (mL)	OD-1 (anti-sera)	OD-2 (anti-sera)	OD (anti-sera) Avg	Protein conc (μg/mL)	Total anti-sera protein (μg)	IFCC EURM-017 (μg/mL)[a]
S1 RBD	0.825	0.29	0.287	0.289	211	174	17.4
N protein	0.943	1.038	1.068	1.053	769	725	72.5
S1	0.932	0.266	0.254	0.260	190	177	17.7
S2	0.935	0.432	0.437	0.435	317	297	29.7
S1/S2 (full-length S)	0.963	0.48	0.491	0.486	354	341	34.1

[a] Concentration in 10 mL starting volume of EURM-017; Avg: Average; S: Spike protein; S1: Spike protein domain 1; S2: Spike protein domain 2; S1 RBD: S1 receptor binding domain; S1/S2: S1/S2 (full-length S); N protein: Nucleocapsid protein. To convert μg/mL to Système Internationale (SI) units (g/L), divide by 1000.

Table 3
Anti-serum concentration at each dilution prepared for each of the five affinity purifications used for PRNT.

Anti-sera specificity	Anti-sera stock (μg/mL)[a]	Concentration at each anti-serum dilution (μg/mL)					
		1:50	1:100	1:200	1:400	1:800	1:1600
S1 RBD	211	4.21	2.11	1.05	0.53	0.26	0.13
N protein	769	15.37	7.69	3.84	1.92	0.96	0.48
S1	190	3.80	1.90	0.95	0.47	0.24	0.12
S2	317	6.34	3.17	1.59	0.79	0.40	0.20
S1/S2 (full-length S)	354	7.09	3.54	1.77	0.89	0.44	0.22

[a] To convert μg/mL to Système Internationale (SI) units (g/L), divide by 1000.

Table 4
Percent plaque reduction at each dilution for each of the five affinity purifications, the $PRNT_{50}$, $PRNT_{90}$ titers, and the protein amount required for $PRNT_{50}$ and $PRNT_{90}$.

Ab specificity	IFCC EURM-017 (μg/mL)[a]	Ab required $PRNT_{50}$ (μg/mL)	$PRNT_{50}$titer	Ab required $PRNT_{90}$ (μg/mL)	$PRNT_{90}$titer	Percent plaque reduction at each antibody dilution					
						1:50	1:100	1:200	1:400	1:800	1:1600
S1 RBD	17.4	0.207	1017	1.01	209	96%	97%	92%	80%	55%	37%
NP	72.5	19.7	39	284.8	2.7	32%	11%	−10%	−20%	−4%	NA
S1	17.7	0.103	1846	0.49	389	100%	100%	94%	97%	69%	58%
S2	29.7	0.893	355	4.95	64	93%	76%	69%	51%	31%	0%
S1/S2 full-length S	34.1	0.287	1234	1.07	333	100%	99%	94%	79%	76%	35%

[a] To convert μg/mL to Système Internationale (SI) units (g/L), divide by 1000.

was greatest for antibodies directed against N protein, despite the greater than two-fold starting concentration. This indicated that neutralizing activity was elicited predominantly against S-related antigens. Neutralizing activity ($PRNT_{50}$ and $PRNT_{90}$ titer) was greater for anti-serum directed against S1 than S1 RBD despite the same protein

Fig. 1. Standardization of assays for detecting different antigen-specific antibodies, S1 RBD (sCOVG and COV2T) and N protein (Roche N protein). **A. S1:** $y = 0.85x − 0.19$, $R^2 = 0.996$; **S1 RBD:** $y = 0.75x-0.10$, $R^2 = 0.9997$; **S1/S2:** $y = 0.30x + 0.00$; $R^2 = 0.999$; **S2:** $y = 0.01x + 0.02$, $R^2 = 0.822$; **N protein:** $y = 0.01x + 0.00$, $R^2 = 0.702$. **B. S1:** $y = 2.134x + 0.75$, $R^2 = 0.995$; **S1 RBD:** $y = 1.06x + 0.43$, $R^2 = 0.994$; **S1/S2:** $y = 0.60x + 0.52$, $R^2 = 0.781$; **S2:** $y = 0.12x + 0.003$, $R^2 = 0.999$; **N protein:** $y = 0.004x + 0.019$, $R^2 = 0.701$. **C.** Roche N protein: N protein: $y = 0.35x − 1.73$; $R^2 = 0.989$. To convert μg/mL to Système Internationale (SI) units (g/L), divide by 1000.

concentration in EURM-017.

3.3. Standardization of two anti-S1 RBD Ig assays and an anti-N protein assay

Next, the detection capability and specificity of two anti-S1 RBD assays for antibodies in the five anti-sera were compared (Fig. 1). Detection was dose-dependent for each anti-serum and assay, indicating that binding activity (antibodies) in patient serum could be correlated by obtaining index values. For the anti-S1 RBD assays, as anticipated, higher index values were detected for S1 and S1 RBD than for S2 and S1/S2 (full-length S) anti-sera. Binding activity was not detectable for anti-serum directed against N protein for anti-S1 RBD Ig assays, but binding activity was dose dependent for the anti-N-protein Ig assay (100 μg/mL to 0.78 μg/mL) (Fig. 1C, Supplemental Table 1).

Additionally, non-purified EURM-017 serum was tested using the sCoVG assay to compare with the yield of the S1 RBD anti-serum in Table 2. The EURM-017 sCOVG result was 15.05 Index which was calculated to be equivalent to 20.2 μg/mL (using the equation for S1 RBD in Fig. 1A, $y = 0.75x-0.10$). This result was not far off that for the S1 RBD anti-serum (17.3 μg/mL) (Table 2). Using the equation for S1 from Fig. 1A ($y = 0.85x-0.19$), 15.05 Index was calculated to be equivalent to 17.93 μg/mL, very similar to the concentration of the S1-anti-serum (17.7 μg/mL) (Table 2).
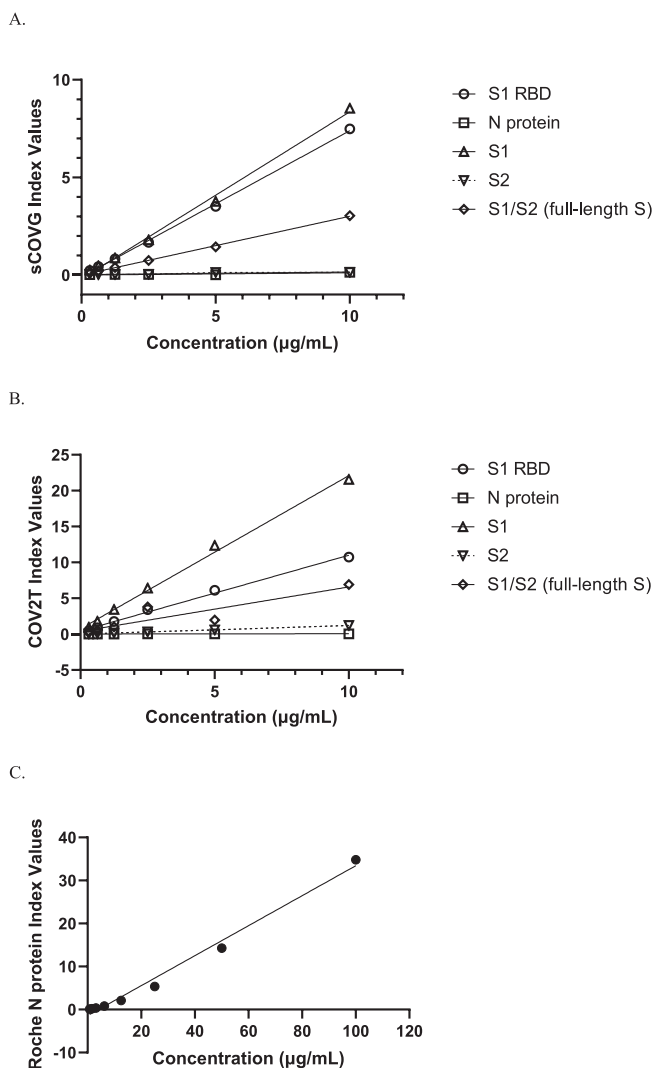
3.4. Neutralization activity ($PRNT_{50}$) in patient samples and correlation with anti-RBD IgG serology

Next, the extent to which results for an anti-RBD IgG assay reflected the presence in serum of antibodies with ability to neutralize SARS-CoV-2 was evaluated. Good correlation was observed between neutralization activity ($PRNT_{50}$) and index values for the anti-S1 RBD IgG assay (sCOVG) assay (Pearson correlation coefficient was 0.84). The index values were converted to EURM-017 using the equation $y = 0.75x − 0.10$, generated in Fig. 1 for S1 RBD anti-serum and the sCOVG assay (Fig. 2).
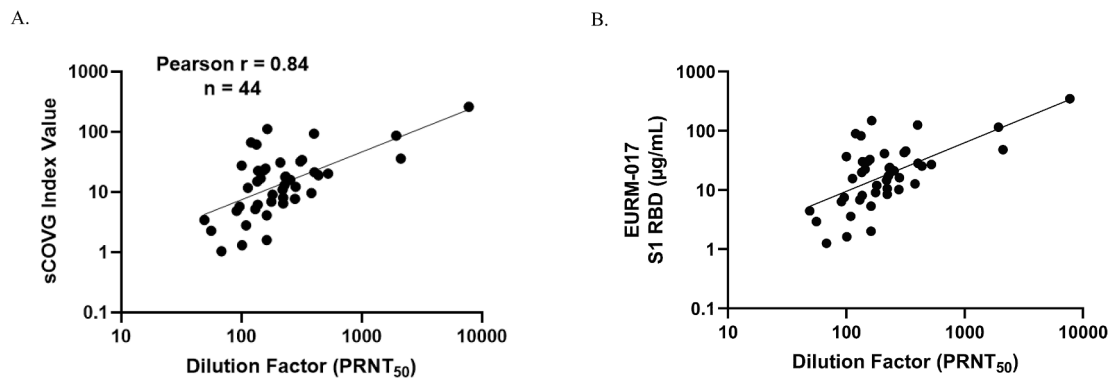
4. Discussion

Currently, there is a paucity of standardized methods and materials for SARS-CoV-2 serology testing [27]. Standardization will allow comparison of results from a range of lab-developed and commercial assays used globally and in the literature. This study reports several new findings with respect to the EURM-017 international standard human serum containing anti-SARS-CoV-2 antibodies. First, we have affinity purified and measured the protein content of five SARS-CoV-2 antigen-specific serum fractions present in EURM-017. Characterizing the five serum fractions in EURM-017 can help produce a standardized procedure, specifically for different assays that detect the same immunoglobulin class and antigen specificity. This will allow comparison of results across different studies and assays around the world.

Second, we have demonstrated that the bulk of neutralization activity resided in the S1-related anti-sera, supporting reports that

Fig. 2. A. Correlation between $PRNT_{50}$ values and index values of the Atellica IM sCOVG Assay (Pearson correlation coefficient was 0.84). The correlation was performed for 44 samples from COVID-19 patients collected several weeks after positive SARS-CoV-2 RT-PCR test results. B. Index values were converted to EURM-017 values (µg/mL) for S1 RBD assays using the equation $y = 0.75x - 0.10$, taken from Figure 1 S1 RBD IgG assay index values versus anti-serum to S1 RBD µg/mL.

antibodies to N protein had much lower levels of, or lacked, neutralization efficacy [3]. Despite a similar starting concentration, neutralization activity of anti-serum directed against S1 was twice that of S1 RBD. This likely reflected the capture of additional neutralizing antibodies in the purification procedure by S1 (to S1 RBD and/or non-S1 RBD sites) versus S1 RBD antigen. It has been reported that reactivity of patient sera was greater against the full-length S than S1 RBD [32], and that neutralizing antibodies target non-S1 RBD domains of S that include the S1 N-terminal domain [9,10] and S2 [11,12]. Here, antibodies directed against S2 in S2 anti-serum likely demonstrated neutralizing activity by blocking virus fusion and RNA entry into cells. Others have reported the presence of neutralizing antibodies to S1 RBD and non-S1 RBD sites [33]. Whether S1 RBD is essential for neutralization by antibodies to non-S1 RBD, S1, and/or S2 was not known in this study. However, Chen et al. [11] showed that sera with S1-specific neutralization did not neutralize after depletion of antibodies to S1 RBD. Here, the $PRNT_{50}$ dilution for anti-serum to S2 was about three to five times lower than dilutions for anti-sera directed against S1 RBD and S1, respectively (despite the greater initial concentration); and, about 3.5 times lower than the dilution of anti-sera directed against S1/S2 (full-length S) (despite the slightly lower concentration). Possibly, not all species that bound S2 antigen were neutralizing, and/or that antibodies directed against S2 included those with lower neutralizing activity. Taken together, the results indicate that anti-sera directed against N protein and S2 were associated with lower neutralizing activity than anti-sera directed against S1-related antigens in EURM-017.

Third, a standardization method for EURM-017 was established for two common anti-S1 RBD Ig assays (compatible with most vaccines based on S protein) [19,20,22–25,34] and an anti-N antibody assay (important for ruling in/out new and past infections in vaccinated individuals). Assays that measure any of the five antigen-specific anti-sera in EURM-017 can now be standardized to the concentration in the reference material as determined in this report. Standardization will not only allow confidence when comparing results across different assays but may help better define thresholds for immunocompetence in COVID-19 patients. Recent studies have shown progress towards defining immune thresholds for protection. Using vaccine trial data, one study found a high correlation between anti-RBD IgG and neutralizing antibody titers [35]. Other studies showed correlations between neutralizing antibodies and vaccine efficacy, and/or between binding antibodies and vaccine efficacy [36–38]. When evaluating results from seven different vaccine Phase III trials, a positive relationship between vaccine efficacy and neutralizing and antibody (S protein IgG) titers was found [37]. Another study showed that vaccine efficacy against symptomatic COVID-19 infection rose with increases in neutralization activity and binding anti-RBD IgG levels [36]. Defining immune thresholds for protection would benefit the development of public health policy for vaccinated and infected individuals and, for selection of convalescent

plasma. Importantly, defining immune thresholds for protection might serve as proxies for neutralization and vaccine efficacy.

In the present study, notably, anti-S1 RBD binding activity as reflected by index values will be different depending on whether the assay targets IgG or Total Igs (e.g., a 10 µg/mL concentration of S1 RBD anti-sera will yield 7.48 index values using the sCOVG assay and 10.73 index values using the COV2T assay.

Using the anti-S1 RBD IgG (sCOVG) assay, higher index values were obtained for the anti-sera directed against S1 and S1 RBD than S2 and S1/S2 (full-length S), consistent with the presence of anti-S1 RBD antibodies. The slightly higher index values for anti-serum against S1 than S1 RBD may have been due to the capture of additional anti-RBD IgG antibodies by S1 antigen in the purification (possibly related to differences in how the epitopes were presented and/or related to capture of antibodies that bridge S1 and S1 RBD) [33]. Almost all the binding activity detected in the anti-serum against S1 appeared to reflect the presence of anti-S1 RBD IgG antibodies, demonstrating the specificity of the assay.

IgG and IgM antibodies represent 75% and 10% of all antibodies in serum, respectively. Whereas the sCOVG assay index values were about the same for S1 RBD and S1 anti-sera, the COV2T index values at each dilution were double for S1 anti-serum. This might have been due to differences in the detection reagent for each type of assay. The COV2T assay detection reagent is the antigen S1-RBD that detects all anti-S1 RBD IgG antibodies (IgG1, IgG2, IgG3, and IgG4) and anti-IgM antibodies, whereas the detection reagent for the sCOVG assay is an anti-human mouse monoclonal IgG antibody that detects IgG1, IgG2, IgG3, and IgG4. Thus, for COV2T, most of the difference in detection between antibodies to S1 RBD and S1 was potentially accounted for by additional purified anti-S1 IgG and IgM (and possibly IgA). However, concerning IgM and IgA, it is unlikely that these antibodies made a significant contribution because several studies have shown that they decline substantially by six weeks after the onset of symptoms—a time when sample collection began for EURM-017 [3,39]. Whether the COV2T assay detected IgG antibodies to S1 additional to those detected for S1 RBD needs to be explored in future studies. The results showing about twice the binding activity in anti-sera directed against S1 as S1 RBD are supported by the findings mentioned above, that neutralizing activity (antibody) was likely associated with both S1 and S1 RBD sites [9,10,32]. Double the binding activity in S1 anti-sera appears to reflect twice the neutralization capacity of S1 versus S1 RBD anti-sera (i.e., the $PRNT_{50}$ concentration for S1 anti-serum was half that required for S1 RBD anti-serum). This suggests that results for the COV2T assay reflected the presence in EURM-017 of antibodies with ability to neutralize SARS-CoV-2; however, more studies are needed to confirm this observation.

Finally, index values for an anti-S1 RBD IgG (sCOVG) assay and neutralization activity ($PRNT_{50}$) in patient sera were well correlated; S1

RBD is reported to harbor about 90% of the neutralizing activity in patient sera [8], and is a proven target for vaccines, therapeutic antibodies, and assays. As mentioned, several studies have shown correlations between various anti-S and anti-S1 RBD IgG assay values and neutralization antibody titers [7,11,13–18]. Our results are consistent with those findings and support the use of the sCOVG assay as a correlate for the presence of neutralizing activity in symptomatic patients. The results are also consistent with those obtained in a different population: A good correlation (Spearman rho = 0.843) between the ADVIA Centaur SARS-CoV-2 IgG (sCOVG) assay and $PRNT_{50}$ values was obtained [40]. (Notably, the Atellica IM sCOVG and the ADVIA Centaur sCOVG assays have the same reagent formulations.) Here, we have converted patient index values to EURM-017 µg/mL values, thereby standardizing our anti-S1 RBD IgG assay results to the EURM-017 S1 RBD anti-serum reference material. The same method can be used for assays based on other antigens and immunoglobulin class. Standardizing other manufacturer assays to EURM-017 µg/mL values will allow comparison of results across assays and manufacturers.

One limitation of this study is that we do not know the relationship of the S1 and S1 RBD neutralizing activity in purified serum fractions, or the nature of the binding activity in S1 and S1 RBD fractions detected with the COV2T assay. Another limitation is that the $PRNT_{50}$ assays were performed with serum from a relatively small number of subjects; nevertheless, results of this study are supported by the independent report mentioned above [40]. Finally, additional studies with a greater number of subjects are needed to further understand the heterogeneity of antibody responses to different viral proteins.

5. Conclusion

Five anti-SARS-CoV-2 antibody fractions present in EURM-017 were affinity purified, quantified, and their neutralization activity ($PRNT_{50}$) compared. Methods were provided for standardizing anti-SARS-CoV-2 assays. Anti-SARS-CoV-2 assays that are based on the detection of any of the five antigen-specific anti-sera present in EURM-017 can now be standardized to the concentrations of the specific anti-serum fraction in EURM-017, as determined in this study: S1 (17.7 µg/mL), S1 RBD (17.4 µg/mL), S1/S2 (full-length S) (34.1 µg/mL), S2 (29.7 µg/mL), and N protein (72.5 µg/mL). The sCOVG assay and neutralization activity in patient sera were well correlated, warranting further exploration of sCOVG (index or standardized µg/mL EURM-017) values as possible surrogates for cumbersome PRNT assays. Standardization of different serology assays to EURM-017 antigen-specific anti-sera will provide confidence when comparing results across studies that use a variety of lab-developed and commercial assays around the globe.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

JF, KO, JC, VS are full-time Siemens Healthineers employees and are stock owners. BAJ, NEB, DF, VDM, and PVA are UTMB employees who had a subcontract with Siemens Healthineers to cover $PRNT_{50}$ sample processing cost. However, the funders had no influence over the results or interpretation. VDM has a patent pending SARS-CoV-2 infectious clone developed at UTMB and the technology has been licensed to several companies; he is on the American Lung Association Advisory Board.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinbiochem.2021.12.009.

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