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Protocols

# Comparative study between virus neutralisation testing and other serological methods detecting anti-SARS-CoV-2 antibodies in Europe, 2021

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ABSTRACT

One consequence of the ongoing coronavirus disease pandemic was the rapid development of both in-house and commercial serological assays detecting anti-SARS-CoV-2 antibodies, in an effort to reliably detect acute and past SARS-CoV-2 infections. It is crucial to evaluate the quality of these serological tests and consequently the sero-epidemiological studies that are performed with the respective tests. Here, we describe the set-up and results of a comparative study, in which a laboratory contracted by the European Centre for Disease Prevention and Control offered a centralised service to EU/EEA Member and pre-accession Member States to test representative serum specimens with known serological results, with the gold standard technique (virus neutralisation tests) to determine the presence of neutralising antibodies. Laboratories from 12 European countries shared 719 serum specimens with the contractor laboratory. We found that in-house serological tests detecting neutralising antibodies showed the highest percent agreement, both positive and negative, with the virus neutralisation test results. Despite extensive differences in virus neutralisation protocols neutralisation titres showed a strong correlation. From the commercial assays, the best positive percent agreement was found for SARS-CoV-2 IgG (sCOVG) (Siemens - Atellica IM Analyzer). Despite lower positive percent agreement of LIAISON SARS-CoV-2 TrimericS IgG kit (Diasorin Inc.), the obtained results showed relatively good correlation with neutralisation titres. The set-up of this study allowed for high comparability between laboratories and enabled laboratories that do not have the capacity or capability to perform VNTs themselves. Given the variety of in-house protocols detecting SARS-CoV-2 specific neutralising antibodies, including the virus strain, it could be of interest to select reference isolates for SARS-CoV-2 diagnostic to be made available for interested EU Member States and pre-accession countries.

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## 1. Introduction

The global effort to mitigate the ongoing coronavirus disease (COVID-19) pandemic caused by severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) (WHO, 2021a) includes, in addition to various public health strategies and a worldwide vaccination programme (WHO, 2021b; ECDC, 2021), a great laboratory effort to reliably detect acute and past SARS-CoV-2 infections. Immunological markers for past SARS-CoV-2 infections and/or vaccinations against SARS-CoV-2 are used to assess the amount of immunity and remaining susceptibility to SARS-CoV-2 in a population. Sero-epidemiological studies can also help to assess the proportion of asymptomatic cases to guide public health actions. Their implementation is aided by the rapid development of numerous in-house or commercially available serological assays (FIND, 2021). Due to the wide variety of serological tests, techniques and differences in antigenic target and/or differences in types of anti-SARS-CoV-2 antibodies, e.g. total immunoglobulin (Ig), IgG, IgM, IgA and/or neutralising antibodies, it is crucial to evaluate the quality of each serological test and consequently the sero-epidemiological studies that are performed with the respective tests. One method to assess the quality of an assay is by proficiency testing as part of an external quality assessment (EQA) (Fischer et al., 2018; Reusken et al., 2018; Matheeussen et al., 2020; Sung et al., 2020; Kohmer et al., 2021).

A different approach would be to compare various serological tests with the gold standard technique to determine the presence of neutralising antibodies, i.e. virus neutralisation tests (VNTs). At this moment, there are still a lot of questions around the actual protection as well as the duration of immunity that the detected COVID-19 antibodies offer. To answer this, it is key to be able to distinguish antibodies that are able to neutralise the virus from other non-neutralising antibodies. While the correlate of protection is yet still unknown (Perry et al., 2022), the amount of neutralising antibodies, i.e. the virus neutralisation titre (VNT50), is a crucial serological marker. However, due to the differences between serological assays and laboratory procedures, it is usually not possible to directly compare numerical values of the results of different methods. There are efforts to harmonise the numerical outcomes of different serological assays by using the internal serology standards that laboratories can use to calibrate their results to international units (NIBSC, 2020, 2021).

SARS-CoV-2 VNTs have to be performed by highly trained personnel in biosafety level 3 (BSL3) laboratories, as they require the addition of live virus cultures. Currently, also because there is only limited need to do so, not all countries/laboratories in the European Union/European Economic Area countries (EU/EEA) region and the pre-accession countries have the capacity to perform VNTs in BSL3 laboratories.

Here, we describe the set-up and results of a comparative study, in which a laboratory contracted by the European Centre for Disease Prevention and Control (ECDC) offered a centralised service to EU/EEA Member and pre-accession Member States to test representative serum specimens with known serological results, for the presence of neutralising antibodies in VNTs.

In addition, in this study the exact same serum specimens were tested by the submitting and the contractor laboratories, and therefore, it was possible to directly compare the results of (semi-) quantitative tests with VNT titres.

## 2. Materials and methods

### 2.1. Study protocol

Laboratories that were known to ECDC to perform SARS-CoV-2 sero-epidemiological studies in Europe and which were part of an ad hoc sero-epidemiology network jointly set up and hosted by ECDC and the WHO Regional Office for Europe during the COVID-19 pandemic were invited to participate in this study via online meetings and email. Participating laboratories were eligible to share up to 100 serum specimens per laboratory, which they could select by their own judgement. The requirements on the materials were that all serum specimens must have been tested with at least one serological test and ≥ 200 μl of sera was still available for VNT testing by the contractor laboratory. If a laboratory had to thaw the specimens for aliquoting, the preference was to keep the specimens thawed and ship them with cooling packs. If specimens were frozen (≥ −20 °C), they were kept frozen and shipped on dry ice.

### 2.2. Project timeline

While the first invitation was sent on 23 February 2021, laboratories could join at any point during the duration of the study until September 2021. Apart from two batches of specimen shipments, one in May 2021 and one in July 2021, all other specimens were shipped from August to September 2021. The processing time (including result reporting to study participant) for the serum specimens that were received before August 2021 was approximately six weeks. For serum specimens received from August to September the processing time varied from six to 12 weeks.

### 2.3. Virus neutralisation assay performed by contractor laboratory

SARS-CoV-2 VNTs were performed as described previously (Rijkers et al., 2020; Caniels et al., 2021). In brief, serum specimens were heat-inactivated for 30 min at 56 °C. Duplicates of 2-fold serial dilutions (starting dilution 1:10) were mixed with 100 median tissue culture infectious dose (TCID50) of SARS-CoV-2 strain 'hCoV-19/Netherlands/ZuidHolland_10004/2020, D614G' and incubated for 1 h at 35 °C in 96-well plates. Subsequently, 20,000 Vero-E6 cells were added to each well and plates were incubated for 72 h at 35 °C. Plates were scored microscopically for 50 % neutralisation. The VNT50 was defined as the value of the sample dilution that showed a 50 % protection of virus-induced cytopathic effect. Notably, the addition of 100 TCID50 virus to the diluted serum specimens was not considered an additional dilution step, as the total amount of potentially neutralising antibodies present is not influenced by this mixing step, only the volume changes. Since the starting dilution was 1:10, titres ≥ 10 were defined as SARS-CoV-2 seropositive. If the highest serial dilution of a serum, i.e. titre 640 using the standard protocol, was still able to neutralise virus growth, the sample was end-titrated in an additional VNT, using serial dilutions up to 15360.

### 2.4. Serological tests performed by the submitting laboratories

All tests performed by the participating laboratories are listed in Table 1. Commercial assays were performed according to manufacturer's instructions.

Four laboratories performed in-house neutralisation tests. The main variable parameters of the in-house neutralisation tests are summarised in Table 2.

### 2.5. Statistical analysis

To compare performance of the different serological tests with the VNT50s determined by the contractor laboratory, their respective positive percent agreement (PPA) and negative percent agreement (NPA) were calculated. PPA and NPA were used instead of sensitivity and specificity, given the absence of a "gold standard" indicating the true presence of anti-SARS-CoV-2 antibodies.

Spearman correlation test was used to assess correlation of (semi-) quantitative assays with VNT50s determined by the contractor laboratory. Data were analysed in Microsoft Excel (Microsoft Corp., Bellingham, WA, USA) and GraphPad Prism 9 software for Windows version 9.3.1 (GraphPad Software, San Diego, CA, USA). Results with a p-value

**Table 1**
Commercial and in-house tests (n = 858) used by study participants and number of shared serum specimens (n = 719). Two laboratories provided serological results of two different assays for the same set of serum specimens, one for 50 samples and one for 89 samples.

| Serological test details | | Method type | Number of serum specimens (percentage of total) | Number of laboratories using assay |
|---|---|---|---|---|
| Abbott | SARS-CoV-2 IgG II Quant | CLIA/CMIA | 86 (10.0 %) | 2 |
| Diasorin Inc. | LIAISON SARS-CoV-2 TrimericS IgG | CLIA/CMIA | 58 (6.8 %) | 2 |
| Euroimmun | Anti-SARS-CoV-2 ELISA IgG | ELISA | 387 (45.1 %) | 4 |
| GenScript | cPass SARS CoV-2 Neutralisation Antibody Detection Kit | sVNT | 22 (2.6 %) | 1 |
| In-house | n.a. | VNT, MNT | 254 (29.6 %) | 4 |
| Siemens | Atellica IM Analyzer - SARS-CoV-2 IgG (sCOVG) | CLIA/CMIA | 51 (5.9 %) | 1 |

Ab: CLIA: chemiluminescence immunoassay; CMIA: chemiluminescent microparticle immunoassay; ELISA: enzyme-linked immunosorbent assay; (s)VNT: (surrogate) virus neutralisation test; MNT: microneutralisation test

≤ 0.05 were considered statistically significant.

## 3. Results

### 3.1. Study participants and specimens provided

Laboratories from 12 countries, i.e., 10 of 30 EU/EEA countries and two of seven EU pre-accession countries shared in total 719 serum specimen with the contractor laboratory (Fig. 1, Table 1). The 272 serum specimen for which the sampling dates are known were collected between 25 May 2020 and 12 August 2021. Two laboratories tested the submitted serum specimen with two different serological tests (50 samples and 89 samples respectively), resulting in 858 total serological results for assay comparison (Table 1).

For the majority of the tested serum specimens the vaccination status was not known or not indicated (Table 3). The mean age was 47.5 years for the vaccinated, 42.1 years for the non-vaccinated and 48.9 years for the unknown status group (Table 3). While in all three groups the majority of serum specimens were provided by female participants (Table 3).

### 3.2. Assay performance

In total, five commercial assays and four in-house assays were used by the 12 submitting laboratories (Table 1). The different serological tests were based on three method types: enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay/chemiluminescent microparticle immunoassay (CLIA/CMIA) and (surrogate) VNTs. While the ELISA results were all obtained with the same commercial kit (Euroimmun - Anti-SARS-CoV-2 ELISA IgG), participants used three different commercial CLIA/CMIA kits (Abbott - SARS-CoV-2 IgG II Quant; Diasorin Inc. - LIAISON SARS-CoV-2 TrimericS IgG; Siemens - Atellica IM Analyzer - SARS-CoV-2 IgG (sCOVG)). Four laboratories performed in-house VNTs, while one participant used a commercial surrogate neutralisation assay (GenScript - cPass SARS CoV-2 Neutralisation Antibody Detection Kit). Two of the serological tests were used by two submitting laboratories and one serological test was used by four submitting laboratories. Laboratories submitted between 22 and 100 serum specimens for VNT testing (Table 1).

To determine the presence of neutralising antibodies, the contractor laboratory performed VNTs on the same serum specimens that were previously characterised for presence or absence of anti-SARS-CoV-2 antibodies by the submitting laboratory. All VNT titre results < 1:10 were considered negative for the presence of neutralising antibodies. Best positive percent agreement of the qualitative results with the VNT results was found for in-house neutralisation assays performed by four laboratories and for the commercial binding assay by Siemens (Table 4). Other test methods, e.g. GenScript - cPass SARS CoV-2 Neutralisation Antibody Detection Kit, Diasorin Inc. - LIAISON SARS-CoV-2 TrimericS IgG and Abbott - SARS-CoV-2 IgG II Quant had lower PPA, ranging from 70.0 % to 59.3 %. Furthermore, 19.4 % of provided samples (151/777) that were positive for the presence of anti-SARS-CoV-2 antibodies in the analysis performed by the submitting laboratories, were not determined as positive for the presence of neutralising antibodies by the contracting laboratory (Table 4).

The only test method that indicated samples as negative, for which the VNT could detect neutralising antibodies was the Euroimmun- Anti-SARS-CoV-2 ELISA IgG (Table 4), influencing the NPA of this test method. For all six of these samples corresponding respiratory swabs were available, which were taken between 28 and 54 days before the blood sample was taken. All six respiratory swabs from these patients were positive in PCR according to the submitting laboratory. For five of the six samples the measured VNT titres were 1:10–1:20.

### 3.3. Correlation of (semi-)quantitative assays with neutralisation testing

Since in this study the same serum specimens were tested by the submitting and the contractor laboratory, it was possible to analyse the results of (semi-)quantitative tests in relation to the VNT50s (Table 5). Correlation was assessed for all (semi-)quantitative methods and for all

**Table 2**
Protocol differences of in-house neutralisation tests of submitting laboratories.

| Method | Virus – clade or lineage or defining mutations | Cells | | Incubation conditions | | | Titre calculation | |
|---|---|---|---|---|---|---|---|---|
| | | Cell line | Monolayer or suspension: | Temp | $CO_2$ | Time | Cut-off: | Virus-serum mix considered dilution: |
| Contractor VNT | B1.1; D614G (S) | Vero E6 | suspension | 35 °C | 5 % | 3 days | 1:10 | no |
| In-house VNT I | hCoV-19/Turkey/HSGM-1192/2020, EPI_ISL_811143 | Vero E6 | suspension | 37 °C | 5 % | 4 days | 1:8 | yes (1:2) |
| In-house VNT II (Simanek et al., 2021) | hCoV-19/Czech Republic/NRL_9640/2020\|EPI_ISL_626593 | CV-1 | suspension | 37 °C | 5 % | 4 days (+1 day staining) | 1:10 | no |
| In-house VNT III | B1; Spike D614G, NSP12 P323L, NSP15 D219N | Vero E6 | suspension | 37 °C | 5 % | 4 days | 1:4 | no |
| In-house MNT (Haveri et al., 2021) | B: hCoV-19/Finland/1/2020 (GISAID accession ID EPI_ISL_407079) | Vero E6 | suspension | 37 °C | 5 % | 4 days | 1:4 | no |

VNT: Virus neutralisation test; MNT: microneutralisation test; CV-1: Normal African Green Monkey Kidney Fibroblast Cells; Temp: temperature

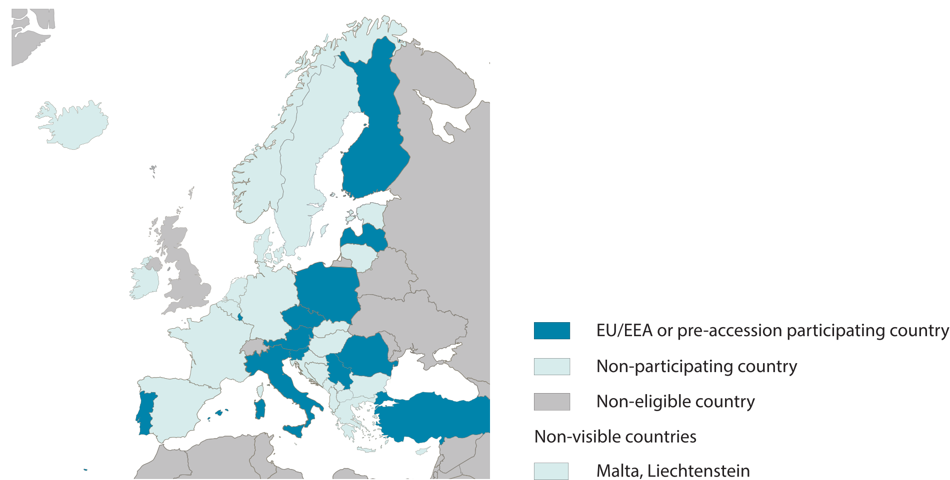


**Fig. 1.** Map of participating laboratories in EU/EEA and pre-accession countries.

**Table 3**
Demographics of submitted sera specimens by vaccination status and summary serological results of contractor laboratory and submitting laboratories.

| | Vaccinated | Non-Vaccinated | Unknown status |
|---|---|---|---|
| Number of specimens | 221 | 97 | 401 |
| Age Mean (SD) [in yrs] | 47.5 (12.6)[a] | 42.1 (11.9)[a] | 48.9 (14.6)[a] |
| Sex | | | |
| Male | 28 (22.4 %) | 27 (29.0 %) | 93 (18.6 %) |
| Female | 51 (40.8 %) | 66 (71.0 %) | 219 (43.7 %) |
| Unknown | 46 (36.8 %) | - | 189 (37.7 %) |
| **Number of specimens:** | | | |
| Tested negative in VNT (%) by contractor laboratory | 69 (31.2 %) | 15 (15.5 %) | 117 (29.2 %) |
| Tested negative in serological tests by submitting laboratories | 12 (5.4 %) | 7 (7.2 %) | 45 (11.2 %) |

a) vaccinated (n = 78); non-vaccinated (n = 93); unknown status (n = 207)

data sets that were end-titrated. Of the seven data sets that could be analysed, three data sets displayed a very strong, i.e., $\rho > 0.8$, significant correlation of results (Table 5). These were two different in-house VNTs and the 'LIAISON SARS-CoV-2 TrimericS IgG' kit (Diasorin Inc.). Serological test methods VNT I and MNT could not be compared because not all titres of these sets were end-titrated. The laboratory that used the commercial assay by Siemens (Atellica IM Analyzer - SARS-CoV-2 IgG (sCOVG)), did not provide the specific (semiquantative) index values, only the qualitative results.

## 4. Discussion

In this study, we compared the results of serological tests that are currently being used in European laboratories with results obtained for the same serum specimens using a VNT performed by a single contracting laboratory. As expected, we found that in-house serological tests detecting neutralising antibodies showed the highest percent agreement, both positive and negative, with the VNT results. Despite the potential for extensive differences in virus neutralisation protocols, the VNT50s that could be assessed also showed strong correlation. This is in line with previous data showing that anti-SARS-CoV-2 neutralising antibody titres have the potential for harmonisation (Nguyen et al., 2021). In general, the contractor VNT detected neutralising antibodies in fewer samples than three of the four in-house VNTs performed by the submitting laboratories. One of the protocol differences that can have an influence on the sensitivity of the methods is the cut-off value of the VNT, i.e. the lowest dilution performed for the VNT. The contracting laboratory used 1:10 as the lowest dilution, while two of the submitting laboratories used 1:4 as their lowest dilution. In the case of a VNT50 of 1:8 the contracting laboratory would not be able to detect claimed neutralisation with the cut-off used. Additionally, the contractor VNT protocol is the only one, using 35 °C for incubation of the plates instead of 37 °C, to simulate a temperature closer to the natural virus replication conditions in the human airways (Eccles, 2021). However, it is not clear whether this would influence the sensitivity of the method. Differences in neutralisation results amongst laboratories could also be due to the virus isolates or cell lines used.

Apart from in-house neutralisation methods, LIAISON SARS-CoV-2 TrimericS IgG (Diasorin Inc.) and cPass SARS CoV-2 Neutralisation Antibody Detection Kit (GenScript) also showed good correlation with

**Table 4**
Percent agreement comparison of serological results by submitting laboratories with VNT results by contractor laboratory per originally performed assay.

| serological test method ↓ | SARS-CoV-2 serology status | | | | Nr of specimens | PPA* [%] | NPA* [%] |
|---|---|---|---|---|---|---|---|
| original serological test submitting lab→<br>VNT50 contractor lab → | positive<br>positive | negative<br>negative | positive<br>negative | negative<br>positive | | | |
| In-house neutralisation tests (VNT, SNT, MNT) | 201 | 39 | 14 | 0 | 254 | 93.5 | 100.0 |
| Siemens - Atellica IM Analyzer - SARS-CoV-2 IgG (sCOVG) | 47 | 0 | 4 | 0 | 51 | 92.2 | n.a. |
| Euroimmun - Anti-SARS-CoV-2 ELISA IgG | 274 | 33 | 74 | 6~ | 387 | 78.7 | 84.6 |
| GenScript - cPass SARS CoV-2 Neutralisation Antibody Detection Kit | 14 | 2 | 6 | 0 | 22 | 70.0 | 100.0 |
| Diasorin Inc. - LIAISON SARS-CoV-2 TrimericS IgG | 39 | 1 | 18 | 0 | 58 | 68.4 | 100.0 |
| Abbott - SARS-CoV-2 IgG II Quant | 51 | 0 | 35 | 0 | 86 | 59.3 | n.a. |

~The original serological test of the submitting laboratory characterised these samples as negative, but the respiratory swabs from these patients were positive in PCR according to the submitting laboratory. The time period between the respiratory swab for PCR testing and time of blood collection for serological testing was 28–54 days.

* PPA: positive percent agreement; NPA: negative percent agreement

**Table 5**
Correlation of reported numerical serological results by submitting laboratory and neutralisation titres determined by contracting laboratory. Table is ordered by correlation (strongest to weakest).

| Serological test method* | Spearman ρ | P value (two-tailed) | Number of data pairs |
|---|---|---|---|
| In-house VNT II | 0.9607 | < 0.0001 | 89 |
| In-house VNT III | 0.9445 | < 0.0001 | 95 |
| Diasorin Inc. - LIAISON SARS-CoV-2 TrimericS IgG | 0.8875 | < 0.0001 | 30 |
| GenScript - cPass SARS CoV-2 Neutralisation Antibody Detection Kit | 0.7952 | < 0.0001 | 22 |
| Diasorin Inc. - LIAISON SARS-CoV-2 TrimericS IgG~ | 0.6512 | 0.0002 | 28 |
| Abbott - SARS-CoV-2 IgG II Quant | -0.2402 | 0.2275 | 27 |
| Abbott - SARS-CoV-2 IgG II Quant~ | 0.1164 | 0.3801 | 59 |

~ Same test as above used by another laboratory.
* Only including (semi-)quantitative methods and data sets for which all entries were end-titrated.

neutralising titres determined by the contracting laboratory. For the cPass kit a comparable correlation to neutralising plaque reduction titres was observed previously (Nandakumar et al., 2021), while the LIAISON kit was not directly compared to a test method detecting neutralising antibodies, but rather to other binding assays (Rychert et al., 2021). Notably, the binding assay with the lowest correlation with VNT results, was SARS-CoV-2 IgG II Quant kit (Abbott). Although other studies find slightly higher correlation with test methods detecting neutralising antibodies (Nandakumar et al., 2021; Ismail et al., 2021), also in these cases the correlation is impacted by the selection of reference assay. The commercial binding assay correlated less well to an in-house PRNT protocol, than compared to cPass SARS CoV-2 Neutralisation Antibody Detection Kit (GenScript) (Nandakumar et al., 2021).

Regarding the commercially available binding assays, the best PPA was found for SARS-CoV-2 IgG (sCOVG) (Siemens - Atellica IM Analyzer), confirming robust performance in earlier studies (Irsara et al., 2021). Despite the lower PPA of the LIAISON SARS-CoV-2 TrimericS IgG kit (Diasorin Inc.), the obtained results showed relatively good correlation with VNT50s. Notably, two different submitting laboratories analysed their serum specimens with the Diasorin assay, therefore two correlations with the contractor VNT could be assessed. The observed difference in correlation showed that other factors could also have an impact on the quantification. Such factors could be the quality of the samples or the handling of material.

Although with the contractor VNT a subset of samples of all serological methods was found to be negative for SARS-CoV-2 antibodies that were indicated by the submitting laboratories as SARS-CoV-2 antibody positive, methods detecting neutralising antibodies showed less discrepancy in this regard. Presumably the difference with other binding methods, e.g., ELISA, could be explained by the detection of only neutralising antibodies with the VNT. The ELISAs performed by the submitting laboratories detected all IgG antibodies specific to S1 of SARS-CoV-2, including those without neutralising capacity (Chen et al., 2022), as they may contain a non-neutralising epitopes.

Six specimens of the tested sera were negative in the serological assay performed by the submitting laboratory and positive in the VNT of the contractor. However, respiratory swabs from these six patients were PCR positive in the submitting laboratory, confirming the neutralisation results of the contractor. The overall low NPA, indicated that all of the assessed serological methods, including ELISA, could be used for population studies as a means to estimate levels of SARS-CoV-2 specific antibodies (Simanek et al., 2021). However, a "calibration" with VNT, e.g. testing of a subset of the studied sera with VNT, would be recommended.

While the set-up of this study allows for high comparability between laboratories and enabled laboratories that do not have the capacity or capability to perform VNTs themselves to assess some of their samples for the presence of neutralising antibodies, there were also limitations in this project. Due to the short time frame of the study, it was not possible to set it up in such a way that all relevant sample characteristics could be shared by all the submitting laboratories. Therefore, it was not possible to compare serological results of vaccinated individuals and results of naturally infected patients. It would also be advisable to request more negative samples from the submitting laboratories for any potential follow-up studies. Given the variety of in-house protocols measuring SARS-CoV-2 neutralising antibodies, which includes among other parameters, also the virus strain used, it would be of interest to select reference isolates for SARS-CoV-2 diagnostic that could be made available for interested EU Member States and pre-accession countries.

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## CRediT authorship contribution statement

RM, JR, KRS, CR, MK and EKB were involved with the study design and study organisation. RM, JR, RG, IC, AH, BH, GK, TN, GP, KP, KPT, JP, MS, RDS, FL, TV, ML, AP and HZ were involved in the testing, with the data collection and analysis. RM, KRS, CR, MK and EKB co-wrote the manuscript. All authors read and approved the final manuscript.

## Declaration of Competing Interest

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

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