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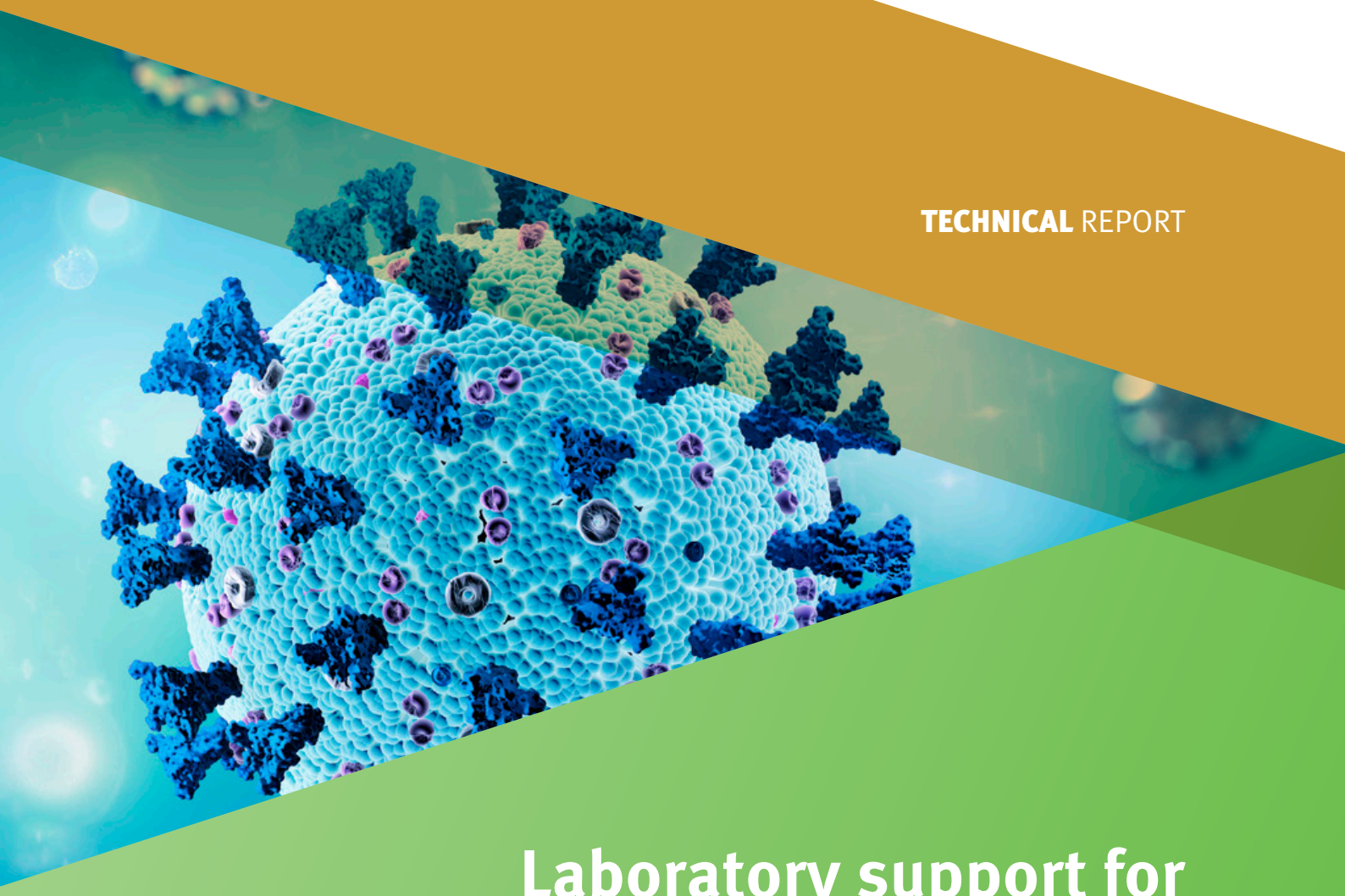

**TECHNICAL** REPORT

# Laboratory support for SARS-CoV-2 virus neutralisation in the EU/EEA - a pilot study

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# Laboratory support for SARS-CoV-2 virus neutralisation in the EU/EEA – a pilot study

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# Abbreviations

| | |
|---|---|
| BSA | Bovine serum albumin |
| COVID-19 | Coronavirus disease 2019 |
| NIBSC | National Institute for Biological Standards and Control |
| OD | Optical density |
| P0, P1, P2, etc, | passage of the virus, used as abbreviation for virus cultures |
| PBS | Phosphate-buffered saline |
| SARS-CoV-2 | Severe acute respiratory syndrome coronavirus 2 |
| TCID | Tissue Culture Infectious Dose |
| TMB, 3,3',5,5' | Tetramethylbenzidine |
| Vero-hSLAM | Vero cells, transfected with plasmid encoding the human SLAM molecule |
| VNT | Virus neutralization test |

# Glossary

| | |
|---|---|
| **Back titration** | A type of titration done in reverse: instead of titrating the original sample, a known excess of a standard reagent is added to the solution and the excess is titrated. |
| **Virus neutralisation assay** | An immunoassay that detects antibody that is capable of inhibiting virus replication, often used in conjunction with infectivity assay. |

# Executive summary

An ECDC pilot study was launched aiming to assist EU/EEA countries with SARS-CoV-2 antigenic characterisation by providing central laboratory testing with advanced methods to six contributing countries. The study was active between March and December 2021. A total of 120 specimens (isolates and nasopharyngeal swabs) were received, 70 of which were successfully sequenced. For the remaining specimens, the sequencing attempts were unsuccessful either due to culturing issues, insufficient material, or technical issues during sequencing. Virus neutralisation assay was successfully performed for 21 specimens selected from different SARS-CoV-2 clades (at least one representative sample of all the clades collected from the participants) and showed varying levels of virus neutralisation among variants. The serum panel contained samples collected during two of the pandemic waves in the Netherlands (when wildtype and Alpha variant respectively were predominantly circulating) and from vaccinated individuals with three different vaccines (Comirnaty, Spikevax and Vaxzervia). The pilot study demonstrated the need for individual optimisation of the assays for each SARS-CoV-2 variant, something that was not previously expected. Moreover, the study also highlighted the added value of having EU/EEA centralised advanced laboratory support for virus characterisation, using standardised methods on specimens deriving from different locations, timepoints and populations.

# Background

The emergence of SARS-CoV-2 and the COVID-19 pandemic were a challenge for existing public health laboratories worldwide, including in Europe. A particular challenge was the timely characterisation of viruses and keeping assays up to date for detecting the quickly emerging SARS-CoV-2 variants caused by multiple pandemic waves worldwide. Knowledge about the virus and technical diagnostic capacities had to evolve with the same rapid pace, to deliver adequate detection and characterisation necessary for driving public health measures.

Several studies have attempted to establish the correlates of protection and the necessary neutralisation levels for protection against SARS-CoV-2 infection [1]. Data from different parts of the world are gradually becoming available and showing the neutralisation levels with different vaccines [2,3]. Given the fast pace of the emergence of new variants and the changing situation regarding vaccination status of the population, obtaining up-to-date data on neutralisation levels from European countries is very important.

An ECDC pilot was launched with the objective of establishing a central laboratory service for EU/EEA countries to submit SARS-CoV-2 viruses or clinical specimens for in-depth antigenic characterisation. Through culturing, sequencing and virus neutralisation assay (VNT), the aim was to characterise viruses collected from different countries and report back to the submitting public health authorities. This was done in order to establish mechanisms and routines to inform the public health authorities at national and international level on the detection of variant viruses that may not be adequately protected against by natural immunity or the current vaccines, and as a result are likely to cause increased number of COVID-19 cases or impact any public health response measures. The established mechanism aimed to ensure that all Member States, particularly those currently lacking sufficient capacity or capability, had access to virus characterisation services of sufficient quality.

# Methods

## Study protocol

Laboratories that performed sero-epidemiological studies in Europe during 2021 were invited from March 2021 onwards to participate in this study via online meetings and emails to the European COVID-19 Laboratory Network and the European COVID-19 seroepidemiology network. Reminders were sent out until August 2021. Participating laboratories were eligible to share either virus isolates or collected nasopaharyngeal swabs by the end of August 2021. If samples were frozen, they were kept frozen and shipped on dry ice.

The shipment was organised as follows: the costs of shipment were covered by the contracting laboratory and instructions were provided to the participating laboratories, i.e. at minimum, a specimen registration form, the biosafety and temperature requirements for the shipment, etc. The participating laboratories registered the specimens electronically by sending an email and providing the shipping information (track and trace). Upon arrival, the specimens were checked and if in good condition, processed further.

The specimens were collected through national surveillance or response systems in place in each country. The specimens could be either representative samples of all positive specimens selected through random sampling, or samples taken because of an unusual epidemiological situation, or viruses initially identified genetically as potential variants of concern that require further testing and more detailed characterisation. The participating laboratories submitting specimens prepared shipment of batches of either:

- virus isolates of previously characterised clinical specimens with known infectious titres for additional characterisation based on virus neutralisation assay on those isolates, or
- original respiratory swabs in virus transfer medium (VTM).

For the successful virus isolates, genetic and antigenic characterisation was then performed.

## Virus culture

Clinical specimens for this pilot study were mainly respiratory specimens (nasopharyngeal swabs) collected by participating laboratories. Viruses were isolated from the nasopharyngeal swabs on Vero-hSLAM cells and if unsuccessful in those cells, then on Vero-E6 cells. After that, the virus was further amplified in one or two passages on Vero-E6 cells. Harvesting was performed at cytopathic effect of >80% at around 72h.

## Sequencing

Sequencing was attempted for all available virus genomes to determine the clade and variations in the spike protein. All virus passages were sequenced and compared to the original virus sequence to exclude viruses that obtained culture-induced alterations.

Total nucleic acid from nasopharyngeal swabs or virus isolates was extracted using MagNApure 96 (MP96) with the total nucleic acid kit small volume (Roche) and eluted in 50 µl Tris EDTA buffer. SARS-CoV-2 specific RT-PCR amplification and sequencing was performed using the Nanopore protocol based on the ARTIC v3 amplicon sequencing protocol [4].

The following modifications were made to the protocol:

- The total volume of the cDNA reaction was 12µl with 0.4 µl Superscript IV[;
- primer concentrations and primer sequence were adjusted (updated primer sequences are available upon request);
- PCR was performed using 47 cycles. Samples were quantified with the Qubit, and samples >35ng/µl were diluted to 6ng/µl in water.

End-prep reaction (5 µl diluted PCR mix) was incubated for 15 min at 20°C and 15 min at 65°C. Barcoding was performed using the NEBNext Ultra II Ligation Module (E7595). Finally, 45ng of library preparation was loaded on a flowcell (Nanopore) and sequencing was performed on a R9.4.1 flow cell multiplexing 48 up to 96 samples per sequence run for a run-time of 30 hours on a GridION (Na-nopore). Consensus genomes were obtained from analysing GridION data with the SARS2seq pipeline and additional manual curation [5]. These genomes were analysed with Pangolin (version 3.1.11) and NextClade (version 1.3.0) to get a final variant call [6].

Sequences were aligned to the Wuhan reference sequence (NC_045512) using MAFFT 7 [7] with the 'addfragments' option. After manual inspection of the alignment a maximum likelihood tree was constructed with IQ-TREE 2 [8], using the GTR+F+I model derived by ModelFinder [9]. Details are available on request.

# Serum panel for antigenic characterisation

For antigenic characterisation of the different viruses, a serum panel was composed. This panel contained seventeen sera or pools of sera including controls. Nine sera from individuals vaccinated twice in 2020-2021 and no history of a SARS-CoV-2 infection were selected (three sera from individuals vaccinated with Comirnaty, Spikevax and Vaxzervia each). Three pools each composed of three sera from Dutch individuals infected with SARS-CoV-2 in the first wave of the pandemic (when the wildtype/index strain was predominant in the Netherlands March-June 2020) were used and an additional three pools, each consisting of five sera from Dutch individuals infected with SARS-CoV-2 from the second wave (when Alpha variant was predominant in the Netherlands after July 2020), were also used. The controls selected for this panel are the NIBSC working standard (NIBSC code 21/234) and one internal control composed of sera from individuals infected with SARS-CoV-2 early in the pandemic. Before use, all sera were heat-inactivated for 30 minutes at 56°C.

# Virus neutralisation assay

Viruses were titrated and the cell culture 50% infectious dose per ml (TCID50/ml) was calculated.

Cultured viruses were tested against the serum panel in a virus neutralisation assay (VNT). The sera were added to a flat-bottom 96-wells plate in duplicate with three-fold serial dilutions starting from 1:10. An equal volume of virus solution was added containing 100 TCID50/well. 100 TCID50/well was also added to six wells without serum and used as virus control wells. The serum/virus mixture was incubated for one hour. Subsequently, 2,5*104 Vero-E6 cells/well were added and incubated for 48 hours. Vero-E6 cells were also added to six wells without serum or virus, as a cell control. All incubations were performed at 35°C and 5% CO2. Additionally, a back titration plate containing ten-fold dilutions starting at a concentration of 100 TCID50/well was used as a control for the amount of virus added to the sample plates.

After incubation, the cells were fixated with 150µl 10% paraformaldehyde and incubated at 40°C for at least 45 minutes before removing the paraformaldehyde and submerging the plates in 70% ethanol. Then the plates were washed with PBS and 100µl per well permeabilization buffer (PBS with 0,02% Triton X-100) was added. Plates were incubated for ten minutes at room temperature (RT). After washing the plates twice with PBS, 100µl per well 1:5000 mouse SARS-CoV-2 nucleocapsid protein monoclonal antibody (in-house produced) in PBS with 1% bovine serum albumin (BSA) was added. Plates were incubated for one hour at room temperature and then washed three times with PBS. As a secondary antibody, 100µl per well of 1:10.000 goat anti-mouse IgG (H+L) highly cross-adsorbed conjugated with HRP (Invitrogen) in PBS with 1% BSA was added and incubated for one hour at RT. Subsequently, the plates were washed three times with PBS. 100µl per well of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added and the plates were incubated for 20 minutes in the dark. The reaction was stopped by adding 100µl per well 0.5M H2SO4. Optical density (OD) values were acquired using an enzyme-linked immuno sorbent assay (ELISA) plate reader and MikroWin software (Siemens).

GraphPad Prism software (Dotmatics) was used to plot the data and calculate the half inhibitory concentration titre (IC50). To normalise the OD values between plates and tests, the average OD value of the virus controls was set as 100% and the average OD value of the cell control at 0%. For samples where no neutralising antibodies could be detected, a titre of <10 was ascribed. The back titration was expected to be within a range of 50 to 200 TCID50.

# Results

## Study participants and specimens

Laboratories from six EU/EEA countries shared 120 virus isolates/clinical specimens with the contractor laboratory (Table 1).

**Table 1. Overview of received materials for central antigenic characterisation testing of SARS-CoV-2 viruses, EU/EEA, 2021**

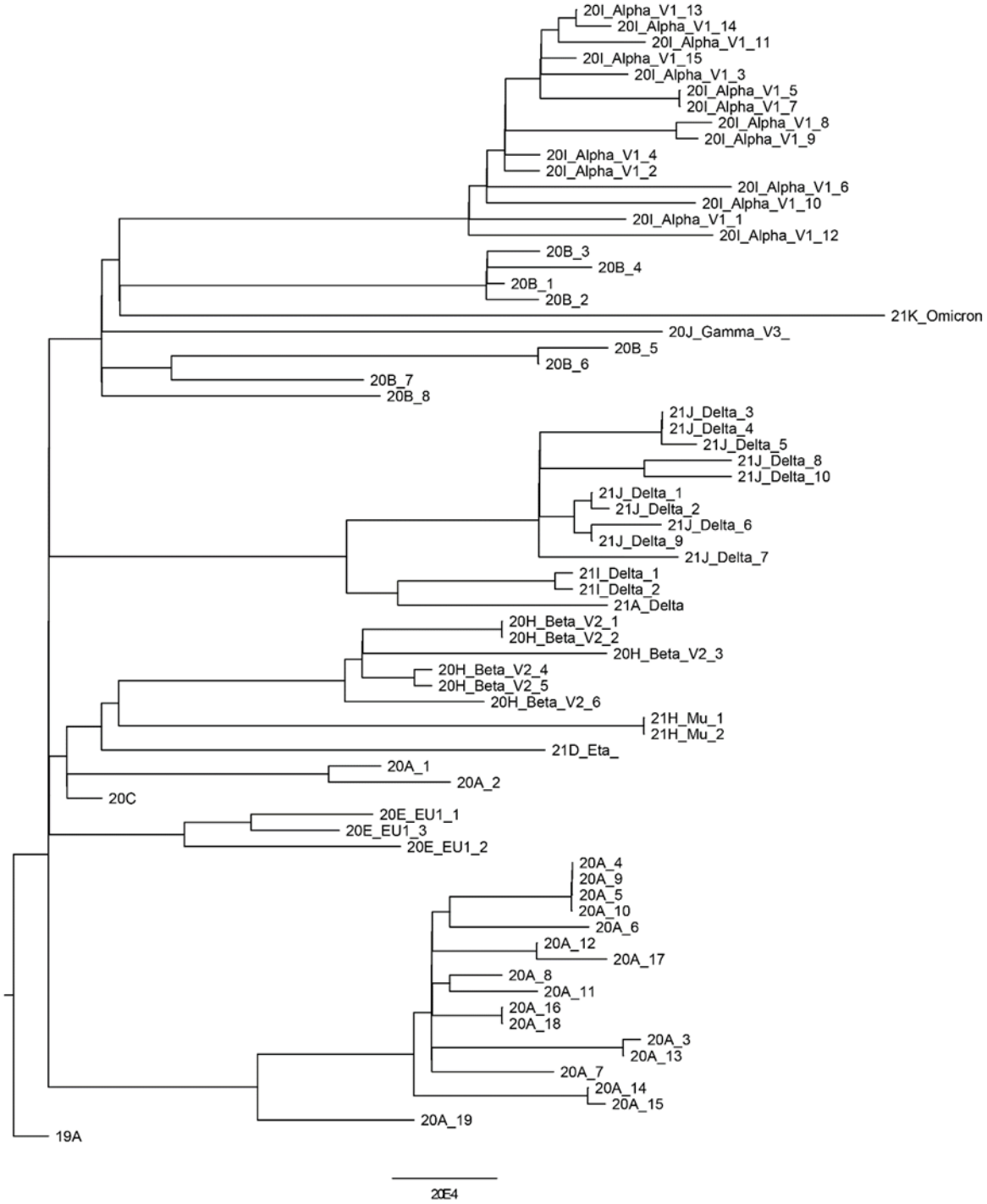
| Country | Lab | Task 6 |
|---|---|---|
| Slovenia | A | 72 nasopharyngeal swabs |
| Austria | B | 15 virus isolates |
| Poland | C | 2 virus isolates |
| Luxembourg | D | 10 nasopharyngeal swabs |
| Greece | E | 20 nasopharyngeal swabs |
| Czechia | F | 1 virus isolate |

In summary, 120 samples were received from six countries, of which 17 were isolates (and one specimen with no infectious material) and 102 nasopharyngeal swabs. In terms of timeline after the invitation in March 2021, 15 virus isolates were received in August 2021, 104 clinical specimens in September 2021 and one virus isolate in December 2021. A total of 70 specimens were successfully sequenced (Table 2, Figure 1). For 48 specimens, the initial P0 virus isolation was unsuccessful and one specimen contained no infectious material. We observed variability during the culturing: different growth times and cytopathic (cytopathogenic) effect were observed with specimens from different SARS-CoV-2 variant clades.

**Table 2. SARS-CoV-2 clade, number of specimens and submitting countries included in the study, EU/EEA, 2021**

| SARS-CoV-2 clade | No of samples | No of submitting countries |
|---|---|---|
| 20A | 19 | 2 |
| 20B | 8 | 4 |
| 20C | 1 | 1 |
| 20E (EU1) | 3 | 1 |
| 20H (Beta, V2) | 6 | 3 |
| 20I (Alpha , V1) | 15 | 3 |
| 20J (Gamma, V3) | 1 | 1 |
| 21A (Delta) | 1 | 1 |
| 21D (Eta) | 1 | 1 |
| 21H (Mu) | 2 | 1 |
| 21I (Delta) | 2 | 1 |
| 21J (Delta) | 10 | 2 |
| 21K (Omicron) | 1 | 1 |

**Figure 1. Phylogenetic maximum likelihood tree of 70 SARS-CoV-2 specimens that were successfully sequenced**




*Specimens that were found to belong to the same clade, were numbered, e.g. 20A_1 to 20A_19.*

A total of six specimens were excluded due to culture-acquired mutation/deletion in spike protein gene. The remaining specimens were divided in subgroups by clade and a sample from each subgroup was randomly selected as representative. The aim was to test at least one specimen from each separate clade. Finally, a total of 23 specimens were further tested by VNT, of which two results were excluded due to discrepancies between the repeats.

Country reports were sent out individually to the participating laboratories, summarising the results of the tests from the specimens submitted.

# Back titration

The back titration of the 21 specimens selected for VNT showed significant variation between specimens (Table 3). No correlation between variant clades and high/low back titration values could be found.

**Table 3. Back titration results of the 21 isolates from different clades**

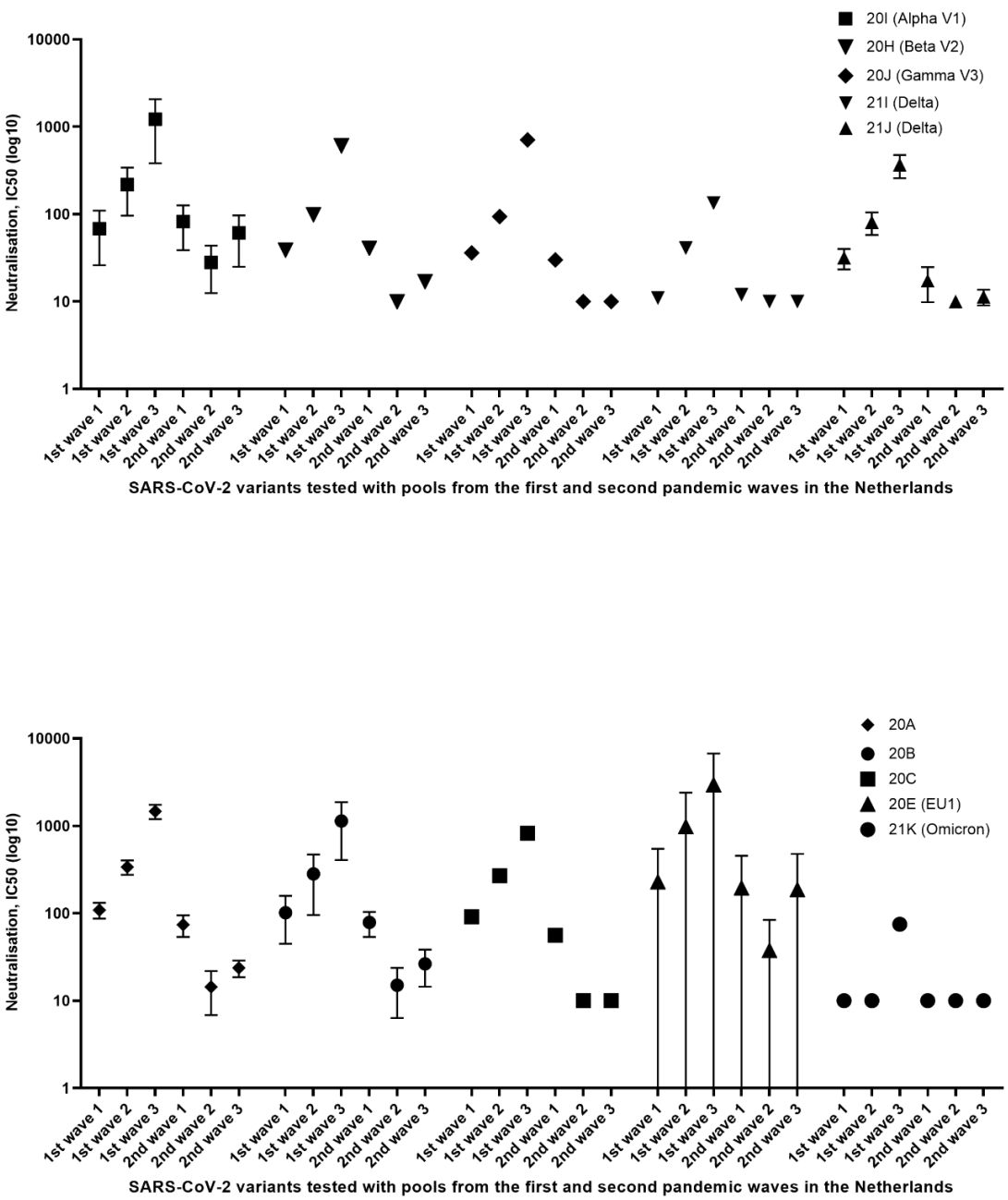
| SARS-CoV-2 clade | No of specimens | Back titration results (up to 3 specimens available) |
|---|---|---|
| 20A | 3 | 383<br>383<br>562 |
| 20B | 2 | 501<br>63<br>32 |
| 20C | 1 | 1 778 |
| 20E (EU1) | 3 | 464<br>316<br>6 |
| 20H (Beta, V2) | 1 | 38 |
| 20I (Alpha , V1) | 3 | 481<br>215<br>383 |
| 20J (Gamma, V3) | 1 | 681 |
| 21I (Delta) | 1 | 3831 |
| 21J (Delta) | 3 | 1 468<br>2 154<br>562 |
| 21K (Omicron) | 1 | 3 162 |

# Virus neutralisation

VNT results by serum panel groups, show specimens of different clades presenting varying degrees of virus neutralisation when tested with the three serum panel specimens from the same waves (Figure 2). Looking into specimens from the first wave (wildtype SARS-CoV-2 in circulation), neutralisation levels varied between <10 and >2000, while for specimens of the second wave (Alpha variant in circulation), neutralisation levels were between <10 and 522. No correlations between specimens from the same country, nor the same variant/clade (Figure 3 and Figure 4) were found.

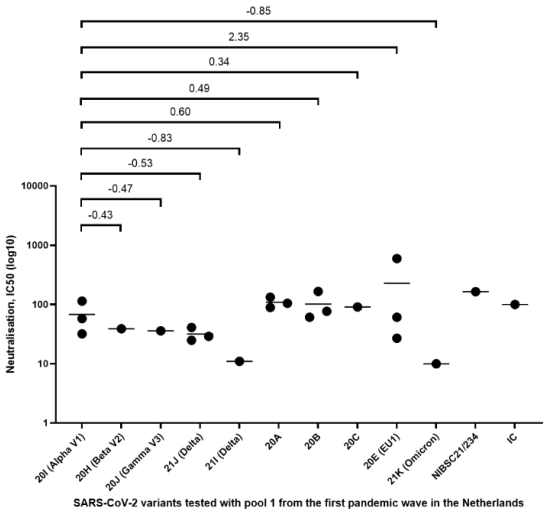
Notably, for this report, the neutralisation titres are not expressed as IU/ml. Multiple standards and controls were taken along each neutralisation test, but they did not show a consistent and reliable pattern of differences in titres.

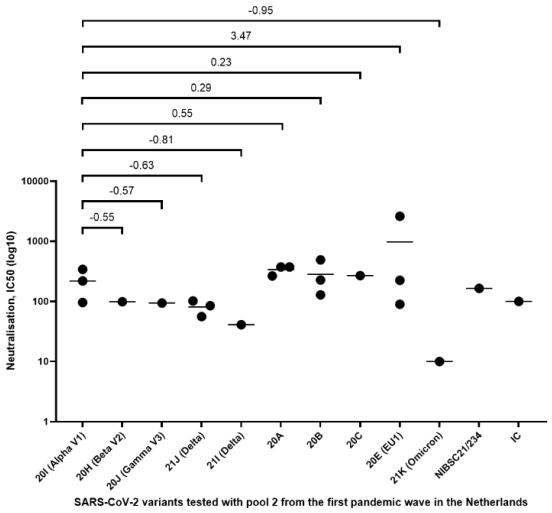
**Figure 2. Summary of VNT assay results for serum panel samples from the first and second pandemic waves in the Netherlands, tested with 21 samples from different clades**

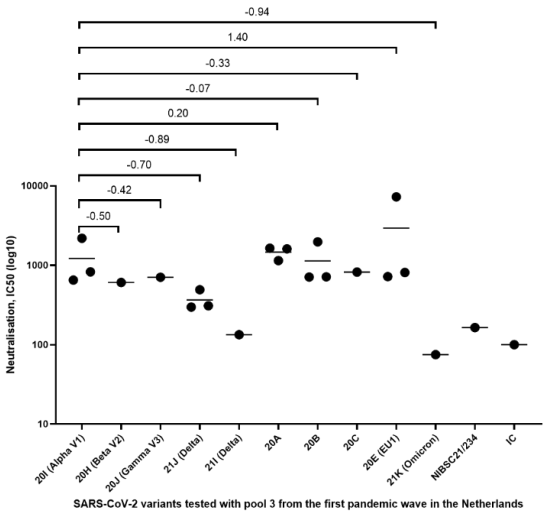



*The upper panel shows clades 20I (Alpha V1), 20H (Beta V2), 20J (Gamma V3), 21I (Delta) and 21J (Delta); lower panel clades 20A, 20B, 20C and 20E and 21K.*

**Figure 3. VNT assay results for three serum panel samples from the first pandemic waves in the Netherlands, tested with 21 specimens from different clades**

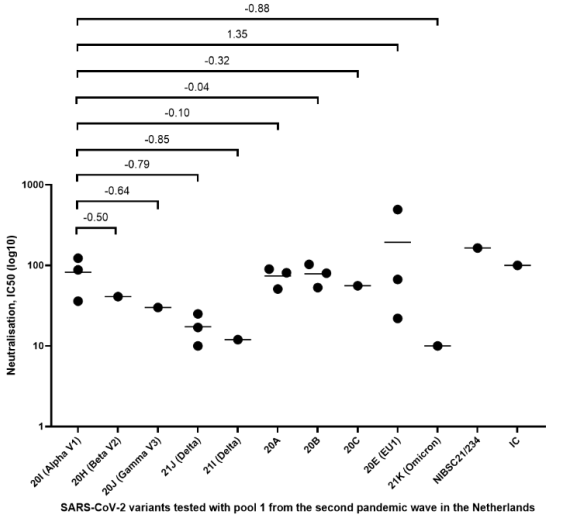


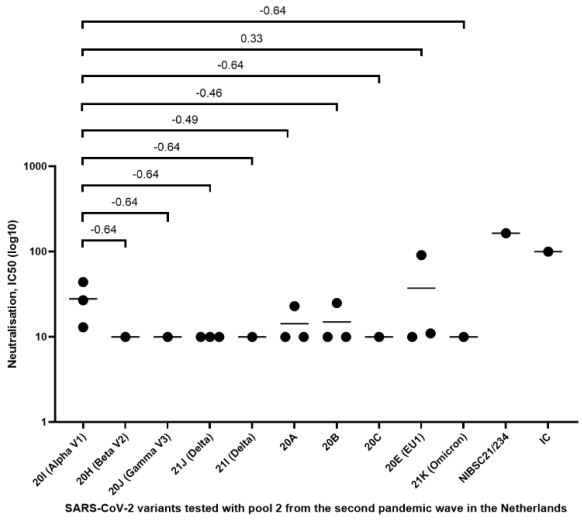



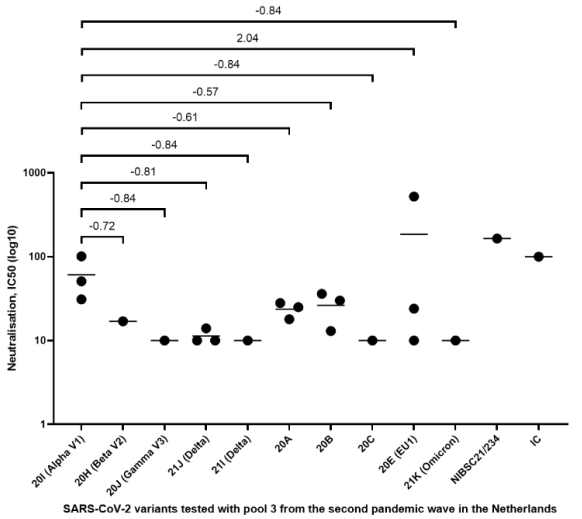




*The fold change compared to the neutralization levels with the Alpha variant is presented above.*

**Figure 4. VNT assay results for three serum panel samples from the second pandemic waves in the Netherlands, tested with 21 specimens from different clades**



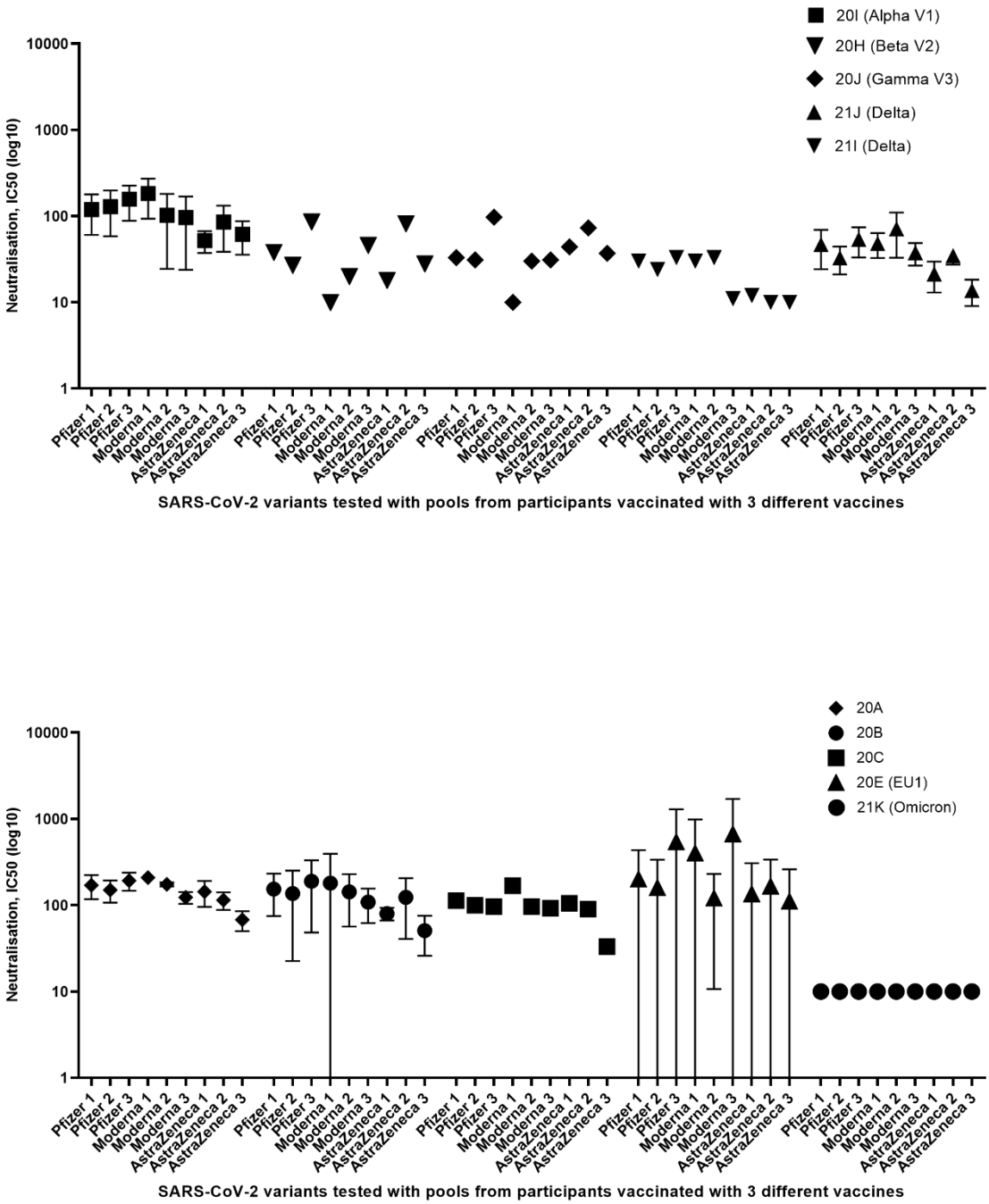




*The fold change compared to the neutralization levels with the Alpha variant is presented above.*

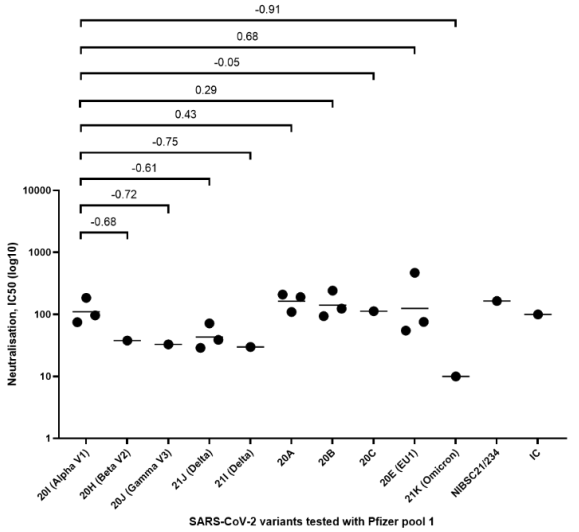
VNT results when using serum panel specimens from patients vaccinated with the same vaccine were more consistent within the same panel group (Figure 5). Apart from an outlier, neutralisation levels varied between <10 and 352 for serum panel samples of patients vaccinated with the Comirnaty vaccine (Figure 6), and between <10 and 423 for those vaccinated with the Spikevax vaccine (Figure 7). Neutralisation levels for serum panel specimens from patients vaccinated with Vaxzervia (Figure 8) were comparable: between <10 and 218 (362 for the outlier sample). Similarly, no correlations between specimens from the same country, nor the same variant were found.

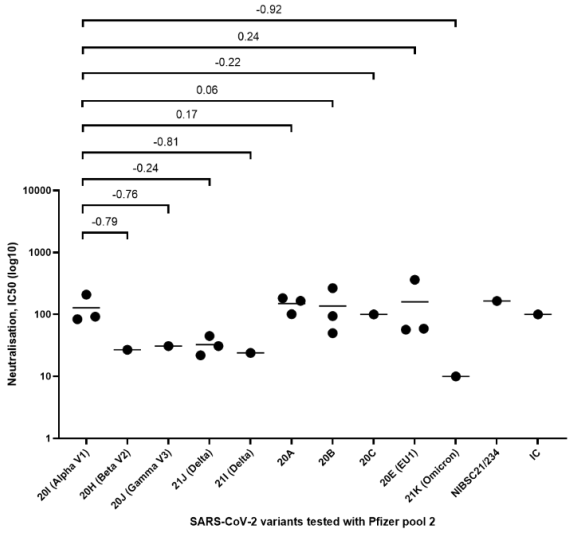
**Figure 5. Summary of VNT assay results for serum panel pools of sera from participants vaccinated with three different vaccines, tested with 21 samples from different clades**

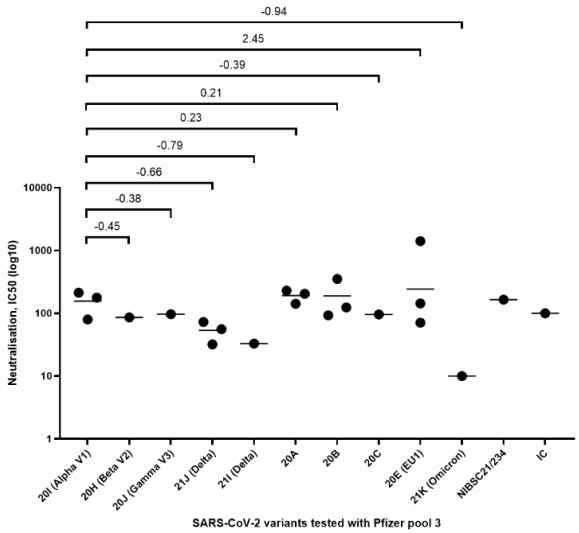



*The upper panel shows clades 20I (Alpha V1), 20H (Beta V2), 20J (Gamma V3), 21I (Delta) and 21J (Delta); lower panel clades 20A, 20B, 20-C and 20E and 21K.*

**Figure 6. VNT assay results for three serum panel pools of sera from participants vaccinated with two doses of the Comirnaty vaccine, tested with 21 specimens from different clades**

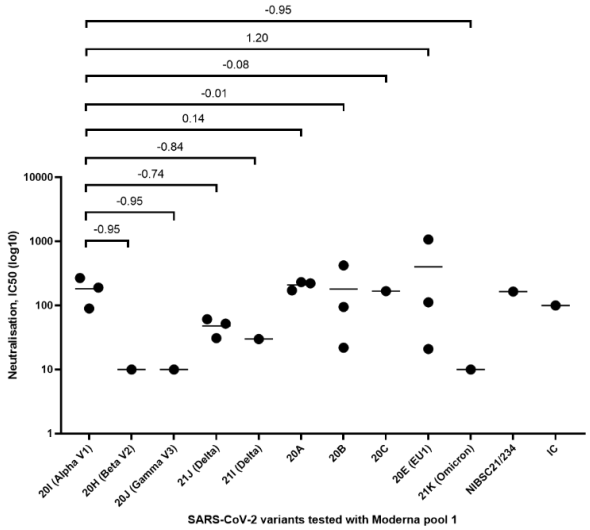


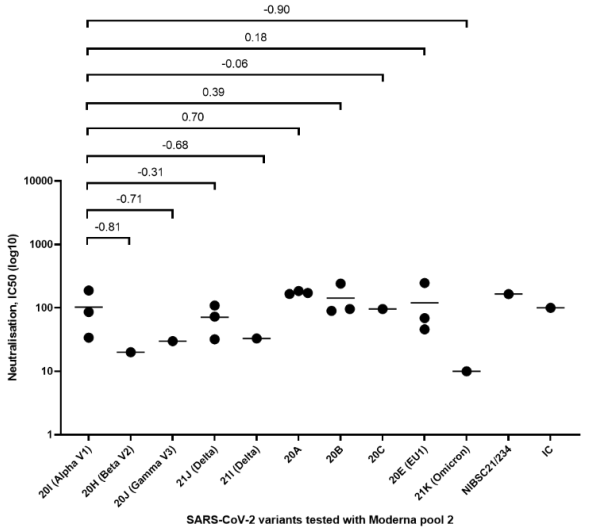



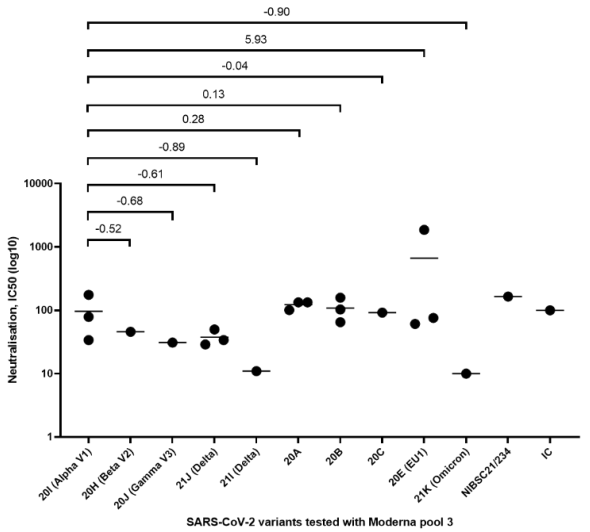




*The fold change compared to the neutralisation levels with the Alpha variant is presented above.*

**Figure 7. VNT assay results for three serum panel pools of sera from participants vaccinated with two doses of the Spikevax vaccine, tested with 21 specimens from different clades**

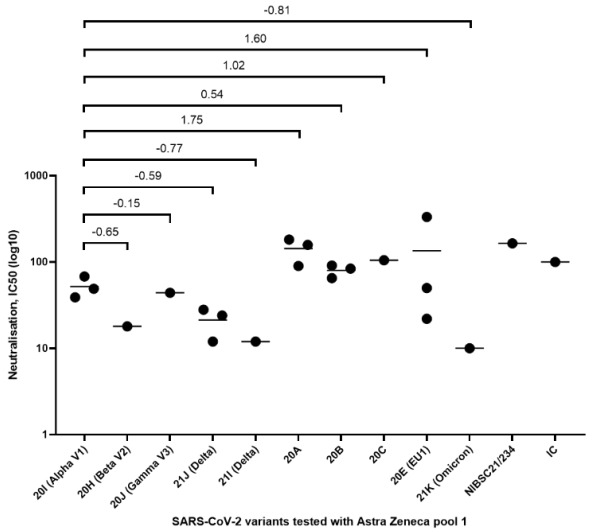


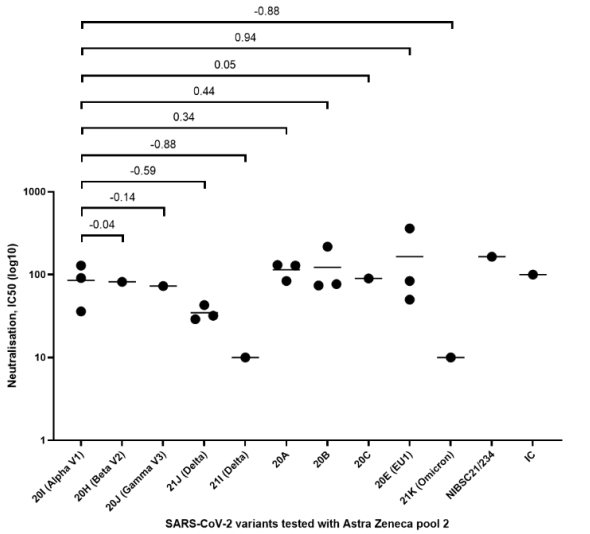



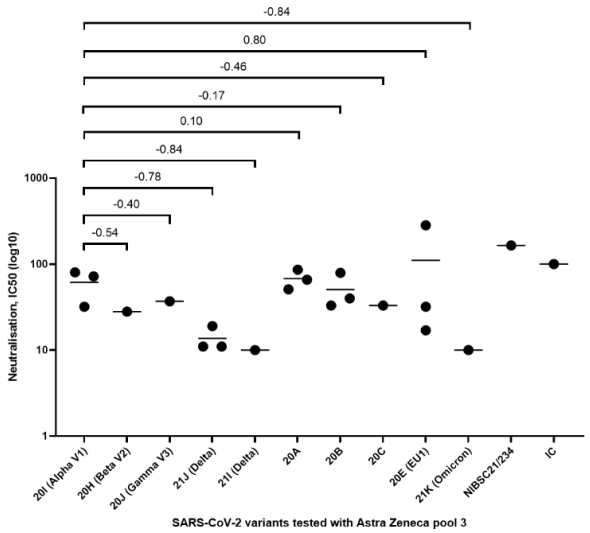




*The fold change compared to the neutralisation levels with the Alpha variant is presented above.*

**Figure 8. VNT assay results for three serum panel pools of sera from participants vaccinated with two doses of the Vaxzervia vaccine, tested with 21 specimens from different clades**




*The fold change compared to the neutralisation levels with the Alpha variant is presented above.*

# Discussion

This project focused on antigenic characterisation of clinical specimens for SARS-CoV-2 variants from EU/EEA countries by a central laboratory service to the national laboratories performing sero-epidemiological studies. The contractor received a total of 120 specimens from six countries and successfully sequenced 70 of them. SARS-CoV-2 culturing and neutralisation proved challenging in this pilot study, revealing the need for individual optimisation for each variant clade, something which was not expected. A total of 21 samples from different SARS-CoV-2 clades were selected and successfully cultured, sequenced and tested via VNT against a serum panel. The degrees of neutralisation varied between samples and clades, and no consistent patterns of correlation, for example between samples from the specimen virus lineage, could be found.

On the wet laboratory side, significant differences between specimens from different variants/clades were observed in terms of growth characteristics during culturing, as well as absolute titres, back titration and VNT results. Using the same protocols, culturing was more prone to failures while cell toxicity in vitro varied. This raised further questions on the need for individual optimisation by variant. A number of specimens were re-titrated and results showed visible variation. These outcomes strongly suggest that individual optimisation is needed for each variant, something that was not yet known in the planning stages of this project. Therefore, additional time for elaborate optimisation tests was not foreseen. Notably, standardisation and normalisation of the assay was also a challenge in another pilot study [10]. Several studies have demonstrated virus characteristics and resistance to neutralisation of the B.1.351, B.1.1.7 and B.1.617 variants [3,11]. Recent studies on the Omicron variant have also shown variation between subvariants, demonstrating a continuous evolution of the virus and increased ability to evade antibodies [2,12,13]. Since neutralisation levels were shown to be highly predictive of immune protection [1], understanding the differences between the antibody's response evoked by wildtype vaccine virus and the circulating variant is extremely important for further vaccine prevention strategies.

Furthermore, a limitation of the pilot study was the lack of a wildtype isolate. Instead, Alpha variant isolates were used, since they were circulating early in the pandemic, and then their neutralisation levels were compared to the fold changes in neutralisation levels for isolates of other clades. In line with results of other studies, the neutralisation levels were much lower against the Omicron variant [14]. Differences between early and later circulating clades were also in line with previously published studies [10,15]. Variations in the spike protein can account for altered virus characteristics and influence neutralising antibodies binding [16,17], as seen in this pilot study as well as in other studies. In such continuous evolving background, special care should be taken to assure optimisation of the assays and usage of comparable approaches across different laboratories.

As a pilot study, this project has helped to outline potential issues with running such a project during a pandemic with a short timeframe, high workload and continuous new emerging variants. We experienced several challenges during the execution of this study. On the organisational side, despite an invitation for participation in March 2021, the main bulk of specimens were not received until the deadline, which was at the end of August 2021, most probably due to high workload and limited human resources that the national laboratories were experiencing during their national pandemic response activities. This led to processing of the whole batch in September, instead of the planned continuous processing in small batches throughout the period March-September. Some transport issues also added additional pressure decreasing the quality of the material received. Future studies should foresee longer transportation times, as well as make sure the cold chain is well-preserved during shipping. Coupled with the pandemic situation and due to the unexpected Omicron variant wave, the processing of the specimens was also delayed due to staff shortages and staff redistribution to urgent tasks.

# Conclusions and potential implications

This pilot study demonstrated the possibility to establish a central testing mechanism for SARS-CoV-2 specimens at EU/EEA level. The central laboratory successfully cultured and sequenced 70 specimens and performed VNT on 21 specimens from different SARS-CoV-2 clades originating from six participating countries. A recommendation for individual optimisation of the culturing and VNT assays protocol for every different SARS-CoV-2 variant was drawn.

On the administrative and logistics side, we recommend better planning for continuous processing of sample batches, both on the central laboratory and the participating laboratories sides. This also includes better organisation of transportation of specimens and buffer times for delays. Care should be taken to space out sample processing so that it can continue even when more capacity is needed elsewhere.

On the laboratory side, dealing with the 'unknown' proved challenging and we recommend additional buffer times to be planned for individual optimisation of assays for each emerging variant, especially when dealing with a changing pathogen in a pandemic scenario.

From a public health perspective, this pilot study highlighted the importance of performing advanced laboratory tests for virus characterisation centrally with standardised methods using specimens deriving from different locations, timepoints and populations. The number of specimens should be sufficient to draw conclusions on neutralisation capability per virus clade, type of sera tested and timepoint in relation to different pandemic waves.

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