Supplementary document 3: Guidelines for laboratory testing

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Contents

Abbreviations	3
Introduction	4
1. Molecular methods	5
1.1 Timing of molecular testing	5
1.2 Specimen types	5
1.3 Sample collection	5
1.4 Sample storage, shipment, transport	6
1.5 RNA extraction and purifications methods	6
1.6 Real-time PCR assay	7
1.7 Interpretation of results	7
1.8 Quality control/accreditation ISO/National standards	7
2. Variant characterisation	8
2.1 Selection of specimens for sequencing	8
2.2 Genetic sequencing of SARS-CoV-2	8
2.3 PCR characterisation of the SARS-CoV-2 variants	8
3. Serology testing	9
3.1 Sample collection and frequency of serological testing	9
3.2 Sample storage	9
3.3 Assay selection	9
3.4 Assay performance	9
3.5 Standardisation of serological assays	9
Annex 1. Table template for sharing sequencing data	10
References	11

Abbreviations

COVID-19	Coronavirus disease 2019
Ct	Cycle threshold
HCW	Healthcare worker
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
RT-PCR	Reverse transcriptase polymerase chain reaction
VE	Vaccine effectiveness
WGS	Whole genome sequencing
WHO	World Health Organization

Introduction

In late 2019, a novel severe acute respiratory syndrome – coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19), emerged. The highly transmissible virus rapidly spread and by 11 March 2020, the World Health Organization declared COVID-19 a pandemic.

SARS-CoV-2 virus is considered a zoonotic pathogen, but the source of the original outbreak is yet to be determined. Infection with SARS-CoV-2 causes illness ranging from asymptomatic, pauci-symptomatic, mild, and severe disease that can result in death. The main symptoms of COVID-19 include fever, a continuous cough, anosmia (loss of smell), and ageusia (loss of taste). Other symptoms include shortness of breath, fatigue, loss of appetite, myalgia (muscle ache), sore throat, headache, nasal congestion (stuffy nose), runny nose, diarrhoea, nausea, and vomiting. Transmission of SARS-CoV-2 can occur through direct, indirect or close contact with infected individuals through either contact with secretions (e.g. saliva or respiratory) or respiratory droplets.

SARS-CoV-2 is a positive sense, single-stranded RNA virus. All four structural proteins, namely the spike (S) glycoprotein, membrane (M) protein, nucleocapsid (N) protein and envelope (E) protein are required for a structurally complete viral particle. The spike (S) glycoprotein binds to the angiotensin-converting enzyme 2 (ACE 2) receptor of the host cell and mediates fusion of the viral and host cell membrane. The N protein is forming the nucleocapsid by binding and packaging the viral RNA genome.

SARS-CoV-2 viral loads have been shown to peak in the Upper Respiratory Tract (URT) within the first week after symptom onset when the risk of transmission to other individuals is considered greatest. Current evidence suggests that individuals become infectious 2-3 days before the symptom onset and remain infectious for up to 10 days after symptom onset, although this period may extend as far as 20 days depending on the severity of disease and the underlying conditions of the host.

The 2019 emerging SARS-CoV-2 strain, usually referred to as the original or 'wild-type' strain has mutated over time. These mutations have given rise to new variants which can exacerbate the risk for global health due to a combination of increased transmissibility, virulence and immune evasion. Variants are categorised as Variants under Monitoring (VUM), Variants of Interest (VOI) and Variants of Concern (VOC) to prioritise monitoring and research. SARS-CoV-2 emerging variants present different clinical and epidemiological characteristics as the virus evolves and gains transmissibility, and evades host protection. There is a strong need to closely monitor the emergence and disappearance of these virus variants.

This document describes the laboratory testing for SARS-CoV-2 infection and the primary outcome of ECDC's prospective multi-country cohort study of hospital-based healthcare workers (HCWs) to evaluate the effectiveness of COVID-19 vaccines in preventing laboratory-confirmed SARS-CoV-2 infection. It should be used by participating laboratories to harmonise testing for SARS-CoV-2 infection with the goal of facilitating the pooling and comparison of data between the study sites.

1. Molecular methods

Laboratory guidance for molecular testing for SARS-CoV-2 infection can be found on the WHO¹ and ECDC² websites, which are regularly updated. In this section, we present a brief summary and considerations for Vaccine Effectiveness, Burden and Impact Studies (VEBIS) HCW vaccine effectiveness (VE) studies.

1.1 Timing of molecular testing

The ideal time point for testing for a possible SARS-CoV-2 infection should be established considering the viral load kinetics. Immediately after infection and during the incubation period the viral load is too low to be detected (1,2). Exponential viral growth during the acute phase of infection leads to peak viral load, which subsequently declines until virus clearance. Quantitative differences in increase and decline of viral load (3) have been reported according to different factors, such as viral variants, vaccination exposure, or host factors. Singanayagam *et al* (3) reported that fully vaccinated individuals with Delta variant infections. A French modelling study (4) using nasopharyngeal samples of individuals infected in 2020 reported that viral clearance occurred earlier in patients aged <65 years (median time of 13 days after symptom onset; IQR: 10 to 15 days)as compared to patients aged \geq 65 years (median time 16 days after symptom onset; IQR: 12 to 20 days). These data support the statement that sampling should be carried out as soon as possible after symptom onset to avoid false negatives.

In the VEBIS HCW study, weekly molecular testing is recommended since HCWs are mostly vaccinated individuals, younger than 65 years and some of them have had multiple infections. Those potentially infected with new variants may therefore experience a rapid decline in viral load.

1.2 Specimen types

There is a consensus among different guidelines that nasopharyngeal samples are the optimal specimen type for detection of SARS-CoV-2 RNA due to easy accessibility and relative high viral loads. The preferred approach would be to perform weekly nasopharyngeal swabbing, if feasible. Nasopharyngeal swabbing is safe when performed by trained health care personnel but is uncomfortable for participants and it is not completely risk free (rare cases of broken swabs or nasal bleeds for example) (5). In addition, personnel training and use of protective equipment results in consumption of health care staff and material resources. Therefore, biweekly nasopharyngeal screening can be considered appropriate if it increases acceptability and engagement of HCWs participating in the study.

Saliva is an alternative sample that can be used if collecting a weekly nasopharyngeal swab is not accepted by participants or for operational considerations (6). Diagnostic sensitivity and specificity for SARS-CoV-2 RNA detection using saliva samples is high (7). Different meta-analyses (8–10) have suggested an overall similar or non-statistically significant lower sensitivity of saliva compared with nasopharyngeal swabs to detect SARS-CoV-2. ACE2 receptors are found in salivary gland cells, and SARS-CoV-2 may infect these cells, therefore the virus can be detected in this fluid. COVID-19 transmission due to saliva-contaminated droplets and aerosols has been extensively documented. Saliva sampling is non-invasive, painless, easy to handle (only a sterile container is needed, with or without virus transport medium), and allows for safe sample collection by health professionals or even self-sampling. Of note, faster clearance of the virus has been reported in saliva than in nasopharyngeal samples. Therefore, if saliva sampling is considered, weekly screening (11) should be preferred to facilitate detection of the virus.

As for saliva sampling, nasal swabbing can only be used for weekly screening in this study. Nasal and throat swabbing can be an alternative to nasal swabbing if the superior virus detection method of nasopharyngeal swabbing is not possible.

1.3 Sample collection

The sample collection is a crucial point related to heterogeneity of diagnostic sensitivity of molecular methods, especially if use of saliva samples is considered.

If a nasopharyngeal swab is used, the guidelines of different centers should be followed. Self-sampling for nasopharyngeal swabbing is not recommended, unless the participating HCWs was previously trained to perform it correctly. Sampling should be performed using personal protective equipment in a ventilated space with

¹ <u>https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance</u>

² https://www.ecdc.europa.eu/en/covid-19/latest-evidence/diagnostic-testing

adequate air renewal. Calcium alginate swabs or swabs with wooden shafts are not allowed for SARS-CoV-2 RNA testing. These swabs can contain substances that inactivate the virus or can inhibit PCR testing. A recent ECDC technical report summarised the different factors related to sample collection, storage, shipment and transport that can affect the sensitivity of saliva as sample type (6). The differences between collection methods make salivary samples a heterogenous category in terms of sensitivity. Saliva can be collected by pipetting directly, with a brush, by oral rinse, coughing, drooling, or spitting. A validation study performed by VEBIS researchers has been conducted using unsupervised spitting, and saliva as sample material is recommended for this VEBIS study (7). Participants should collect their own saliva in the early morning or after a fasting period of two hours to avoid food remains. They should be instructed to spit their saliva into tube collectors, close the tubes with screw caps, decontaminate the external surfaces of the tubes with an appropriate disinfectant³, and identify them with barcode labels. All individuals involved in collecting and transporting specimens should be trained in safe handling practices and spill decontamination procedures.

*Note that although nasopharyngeal self-sampling is not recommended as a routine method, it could be useful in some specific situations such as holidays, in case the participant is unable to come to the hospital, provided that the participant is trained for self-sampling.

1.4 Sample storage, shipment, transport

If a nasopharyngeal swab is used, using a dry collector or placing swabs immediately into sterile tubes containing 2-3 ml virus transport medium, either with or without virus inactivator, is recommended. It is also recommended to avoid the use of inactivating virus transport medium to allow for virus isolation and whole genome sequencing (WGS) for surveillance.

Fasting saliva can be stored directly in a RNase/DNAse-free tube. If the collector tube is not DNase/RNasa-free the sample can be transferred to sterile reaction tubes with disposable Pasteur pipettes. Adequate saliva stability has been reported at room temperature for up to 24 hours, for up to 48 hours at fridge temperature (7) and up to 72 hours in virus transport medium according to the manufactures specifications. Ott *et al* (12,13) reported good stability of fasting saliva storage even beyond 24 hours at room temperature.

The utility of collecting saliva in expensive tubes containing purported stabilisation buffers comes into question. Previous work with saliva samples has shown that some buffers optimised for host nucleic acid stabilisation may actually inhibit viral RNA detection in saliva especially if direct PCR is performed (14). In addition, a dilution factor should be considered when these buffers are used, so an additional concentration step is mandatory to achieve an adequate volume from diluted saliva.

Transport of specimens should be performed according to laboratory guidelines in your country or to WHO laboratory guidelines together with applicable national regulations. International transport of specimens should follow applicable international regulations as described in the WHO Guidance on regulations for the transport of infectious substances 2019–2020 (15).

For each specimen, time of collection, conditions for transportation and time of arrival at the laboratory will be recorded. Specimens should be shipped to the laboratory as soon as possible after collection. If the specimen is not likely to be shipped to the laboratory within 72 hours, it should be frozen, preferably at -80 °C, and then shipped on dry ice. Aliquoting samples prior to freezing is recommended to minimise freeze thaw cycles. Samples should not be refrozen.

The residual samples can be stored in a biobank for future research projects if participants consent. All positive and inconclusive samples and a proportion of the negative samples collected before//during/after any epidemic wave should be stored and used for additional testing as approved under this study.

1.5 RNA extraction and purifications methods

There are different preliminary steps prior to performing the RT-PCR assay. After an inactivation step, the RNA is extracted and purified. To ensure sufficient amount of RNA for amplification, an additional sample concentration step is advisable.

Fasting saliva is a clean sample with low content of PCR inhibitors, so this type of sample can be used directly for PCR after an inactivation-extraction step using heating, as was shown in a VEBIS funded validation study. The direct RT-qPCR workflow involved saliva incubation of the saliva sample in a heat block for 15 minutes at 96°C to maximise virus inactivation and RNA extraction.

All samples stored in non-inactivating VTM must undergo an inactivation step before RNA extraction.

³ Found here, for example: <u>https://cfpub.epa.gov/wizards/disinfectants</u>

1.6 Real-time PCR assay

Molecular tests currently recommended by WHO for the diagnosis and confirmation of SARS-CoV-2 infections are described on a dedicated WHO webpage⁴. For harmonisation purposes in the current VEBIS study, CE-IVD Realtime RT-PCR for specific detection of SARS-CoV-2 is the recommended molecular test. Several commercial SARS-CoV-2 RNA detection assays with CE-IVD marking are available on the market. Information on these assays can be found in the test directory of the Foundation for Innovative New Diagnostics (FIND)⁵ and in the JRC COVID-19 In Vitro Diagnostic Devices and Test Methods Database of the European Commission⁶. Of note, CE-IVDmarked tests should specify the recommended platform for amplification. It is mandatory to use a test with at least two specific targets of SARS-CoV-2 RNA and an internal control to check the quality of the sample. The test must be updated for the proper detection of circulating SARS-CoV-2 variants. The use of Multiplex PCR for SARS-CoV-2 and other respiratory virus is also desirable.

1.7 Interpretation of results

Results are categorised according to the specifications of the manufacturer. For the purpose of the VEBIS HCW study, we consider the following classification: negative (absence of SARS-CoV2 target detection or detection over 38 Ct and detection of internal control), positive (detection of two SARS-CoV-2 target detection under 38 Ct) and inconclusive (only one SARS-CoV-2 target detection).

Inconclusive samples should be repeated for clarification. If an inconclusive result persists, a new sample of the participant should be analysed.

1.8 Quality control/accreditation ISO/National standards

It is highly recommended that laboratories participate in quality control/accreditation programs including the CE-IVD market test used for Real-Time PCR detection.

⁴ <u>https://www.who.int/publications/i/item/WHO-2019-nCoV-lab-testing-2021.1-eng</u>

⁵ FIND website available at: <u>https://www.finddx.org/covid-19/sarscov2-eval-molecular/molecular-eval-results</u>

⁶ JRC dashboard available at: <u>https://covid-19-diagnostics.jrc.ec.europa.eu</u>

2. Variant characterisation

During virus replication, mutations occur frequently in virus genomes. These mutations can often result in amino acid changes, originating new variants. SARS-CoV-2 variants can be classified as Variant Under Monitoring (VUM), Variant of Interest (VOI) or Variant of Concern (VOC), according to the characteristics presented and its consequences to public health. The emergence of new variants can result in increase in transmissibility or in virulence, or decrease in VE, in public health measures effectiveness, in diagnostics sensitivity or in therapeutics effectiveness (16). In this sense, variant characterisation has an important role in VE studies.

2.1 Selection of specimens for sequencing

Positive samples with cycle threshold (Ct) values of <30 should be sequenced for determination of circulating variants. Samples should be shipped to the National Reference Centers for molecular surveillance of SARS-CoV-2 variants according to national regulations. ECDC provides guidance on sequencing methods to detect and identify SARS-CoV-2 variants (17). If not all positive samples with Ct<30 are to be sequenced, those that are sequenced should be randomly selected.

2.2 Genetic sequencing of SARS-CoV-2

There are several methods for sequencing of SARS-CoV-2 (18). Next Generation Sequencing for WGS is considered the best method to characterise SARS-CoV-2 variants, however, specific equipment and bioinformatics analysis expertise are required. To expand the WGS capacity at national and European level, ECDC and WHO supported countries in the implementation of WGS and in the establishment of bioinformatics pipelines for sequence analyses. When it is not possible to perform WGS, at least partial S-gene sequencing covering the entire N-terminal and RBD is recommend to characterise variants properly. Both WGS and partial sequencing can be performed by next-generation sequencing (NGS) or by Sanger sequencing (19).

Public protocols for SARS-CoV-2 sequencing are available (<u>https://artic.network/ncov-2019</u>), and it is mandatory to check for updates in primer sequence regularly, since primer-template mismatches can happen in consonance with the emergence of new variants.

The viral load is an important factor for sequencing success. To better manage the available resources, samples that present Ct values under 25 have higher chances of sequencing success. In some cases, sequencing of samples with Ct values between 25 and 30 is justified.

In this VEBIS project, given the importance of the project and the limited sample size, the sequencing of samples with Ct values under 30 is recommended. It is also recommended to share sequence data in public databases such as GISAID to enable global analysis (<u>https://www.gisaid.org</u>).

In this VEBIS study, the data on the SARS-CoV-2 sequences should be shared with the focal point for sequence analysis, Francisco Pozo, using the table template provided in the Annex 1. Sharing of sequences through the European data portal: <u>https://www.covid19dataportal.org</u> is highly recommended.

2.3 PCR characterisation of the SARS-CoV-2 variants

RT-PCR-based assays are useful tools to characterise known SARS-CoV-2 variants within a few hours. However, confirmation by sequencing is highly recommended. In addition, real-time update on the current epidemiological context as well as a good knowledge on the molecular characteristics of each circulating variant is mandatory to correctly interpret RT-PCR-based assays results (19,20).

There are several nucleic acid amplification tests (NAAT)-based methods to screen SARS-CoV-2 variants. S-gene target failure (SGTF) is useful to detected variants that present deletion at positions 69-70 (Δ 69-70), since variants with this deletion presents weaker or no signal to S-gene target. SGTF occur (but are not exclusively) in Alpha (B.1.1.7) and Omicron (B.1.1.529) sub-lineages BA.1 and BA.5.

Multiplex RT-PCR-based assays and RT-PCR specific for melting curve analysis can also be applied to detect single nucleotide polymorphisms (SNP). Multiplex assays can provide more robust results since several variants can carry the same SPN. In this sense, a combination of different SNP targets is able to better characterise a variant. Caution in interpretation is necessary, and failure in SNP detection can occur even whether the variant carries the SNP target.

SNP RT-PCR have the advantages of a higher throughput for the genetic characterisation of circulating variants and low cost but are not necessarily able to identify new circulating SARS-CoV-2 variants. Whole genome sequencing is still of great value to monitor the genetic characteristics of SARS-CoV-2. Both methodologies, SNP RT-PCR and whole genome sequencing using NGS are complementary for an in-depth characterisation of the circulating SARS-CoV-2 variants.

3. Serology testing

The adaptive immune system is essential for control and clearance of SARS-CoV-2 infection. Antibodies are essential components of the adaptive immune system. The production of specific antibodies against SARS-CoV-2 can be trigged by infection or vaccination. Immunoglobulins type G (IgG) are antibodies that persists longer after an infection or vaccination, being one of the mains targets used when investigating the adaptive immune response (21). In addition, previous data have shown that anti-spike (S) IgG levels are highly correlated with neutralising activity (22,23). However, the correlation between IgG or neutralising antibodies and protection against SARS-CoV-2 infection is not established. This subject is highlighted given the emergence of new variants, which can present substantial antigenic differences, resulting in lower neutralisation capacity, with increased risk of infection in previously infected or vaccinated individuals (24).

Nucleocapsid and spike protein are the main antigens used in serological investigations. Since the vaccines used so far in this study are based on the spike protein, the use of both targets is essential to differentiate adaptive immunity trigged by infection, as infected people will develop anti-nucleocapsid antibodies (anti-N IgG) or by vaccination. However, it is necessary to take into consideration that most, but not all, individuals seroconvert after an infection. The half-life of anti-N IgG in a cohort of HCWs was estimated to be 85 days (26), however, this value varies with age, ethnicity and disease severity (26,27). In this sense, a negative result for anti-N IgG should be interpreted with caution.

3.1 Sample collection and frequency of serological testing

In the VEBIS context, the collection of blood samples is recommended at enrolment, and at least every 6-12 weeks during the follow-up period. Sample collection can occur more often if resources allow. The blood should be collected in dry tube, centrifuged and separation of sera should be performed as soon as possible. Time elapsed between sample collection and test performance should follow the assay recommendation. Serum is the most common sample used in tests but, depending on the assay recommendation, samples of plasma, whole blood, and dried blood spots can also be used in tests.

3.2 Sample storage

Samples should be stored refrigerated ($<8^{\circ}$ C) for five to seven days, until the test is performed, and at -20°C or preferentially at -80°C for an undetermined time, to allow future additional tests if possible.

3.3 Assay selection

There are several assay and different molecular targets to measure the presence of antibodies against SARS-CoV-2. The choice of assay should be based on the purpose.

Regarding binding antibodies against SARS-CoV-2, the main methods available to perform serology tests are lateral flow, Enzyme-Linked Immunosorbent Assay (ELISA) and chemiluminescent immunoassay (CLIA). Lateral flow assays are often used as point of care tests, while ELISA and CLIA are indicated for laboratory use.

Quantitative or semi quantitative assays are preferred to allow to monitor changes in antibody profiles and titres over time and to correlate serological titres with breakthrough infections. Assays that differentiate between S and N targets are to be preferred in the VEBIS project context. In the event total IgG measurements are performed, we suggest that additional methodologies that can distinguish anti-S IgG and anti-N IgG should be performed in collaboration with the National Reference Laboratories.

3.4 Assay performance

Most available assays show a test sensitivity of at least 80%. In the current VEBIS project, using assays with no less than 95% sensitivity and 98% specificity to measure SARS-CoV-2-specific antibodies is recommended.

3.5 Standardisation of serological assays

WHO and NIBSC have standard panels for serology testing against SARS-CoV-2 available for distribution. These standards can serve as a basis for calibrating tests that quantify antibodies and can support the presentation of quantitative results in Binding Antibody Units (BAU/ml). However, even using the same units, we have to be cautious when providing results obtained using different methodologies (28) over time. Whenever possible, each laboratory should use the same methodology during the lifetime of the project.

Participation in EQAs is highly recommended.

Annex 1. Table template for sharing sequencing data

Sharing all sequences obtained from positive samples for SARS-CoV-2 during the project is recommended to enable global analyses. Data should be sent to the focal point for genetic analysis. In this annex, we provide the table template to share the data.

In the column 'LAB ID', the sample identification in the laboratory, which enable the correspondence with the epidemiological data, should be provide.

The 'Public code' corresponds to the identification of the sample in the public platform (GISAID). This code has the followed structure: hCoV- 19/country/lab id/year.

The 'Collection date' corresponds to the date when original sample was collected.

The 'gisaid_epi_isl' corresponds to the number obtained after the submission of the sequence to GISAID. This identification starts with 'EPI_ISL'.

SARS-CoV-2 sequences_Table tem			
LAB ID	Public code	Collection date	gisaid_epi_isl

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