Opinion Paper

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Ad interim recommendations for diagnosing SARS-CoV-2 infection by the IFCC SARS-CoV-2 variants working group

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Abstract: This document, endorsed by the IFCC Working Group on SARS-CoV-2 Variants, aims to update previous indications for diagnosing acute SARS-CoV-2 infection, taking into consideration the evidence that has emerged after the origin and spread of new lineages and sub-lineages of the virus characterized by mutated genetics and altered biochemical, biological and clinical characteristics. These indications encompass the use of different diagnostic strategies in specific clinical settings, such as high risk of SARS-CoV-2 infection (symptomatic patients), low risk of SARS-CoV-2 infection (asymptomatic subjects) at hospital admission/contact tracing, testing in asymptomatic subjects, in epidemiologic surveys and/or population screening, along with tentative indications for identification of new lineages and/or sub-lineages of SARS-CoV-2.

Keywords: COVID-19; diagnosis; SARS-CoV-2; sequencing; variants.

Test indications in specific settings

This document, endorsed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) “Working Group on SARS-CoV-2 Variants”, aims to update previous indications for diagnosing acute SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection, taking into consideration the evidence emerged after origin and spread of new lineages and sub-lineages of the virus characterized by mutated genetic, biochemical, biological and clinical characteristics [1, 2]. These indications encompass the use of different diagnostic strategies in specific clinical settings, such as high risk of SARS-CoV-2 infection (symptomatic patients), low risk of SARS-CoV-2 infection (asymptomatic subjects) at hospital admission/contact tracing, testing in asymptomatic subjects, epidemiologic surveys and/or population screening, along with tentative indications for identification of new lineages and/or sub-lineages of SARS-CoV-2.

The indications regarding the tests and the relative procedures used for diagnosing SARS-CoV-2 infection in specific settings are summarized in Table 1, and are almost overlapping with those previously published by the IFCC Task Force on COVID-19 [3], the World health Organization (WHO) [4], the US Centers for Disease Control and Prevention (CDC) [5, 6] and The European Centre for Disease Prevention and Control (ECDC) [7].

High risk of SARS-CoV-2 infection (symptomatic patients)

The diagnosis of high-risk patients (i.e., those presenting signs and symptoms suggestive for SARS-CoV-2 infection)
should still be carried out with laboratory-based molecular assays, using upper or lower respiratory tracts samples. Considering that patients with symptomatic infection need a timely and accurate diagnosis for lowering the risk of unfavourable disease progression, this approach remains the most appropriate for reducing the risk of false negative test results, which are then associated with delayed treatment and/or isolation [8]. The ideal turnaround time (TAT) ranges between 6 and 12 h and confirmation is not usually needed due to the high positive and negative predictive values of these tests. Nonetheless, the test should always be repeated using a different laboratory-based molecular assay within 24–48 h when the original test is negative in highly suspected patients. A false negative test results may in fact originate for several causes, such as preanalytical inaccuracies and/or mistakes (e.g., inappropriate procedures used for sample collection, transportation, storage and preparation for testing), technical failures, postanalytical errors (i.e., test result interpretation) and so forth, but also for gene target failures caused by new mutations which may no longer be recognized by probe and/or primers uses in some molecular assays [9–11]. The ideal sample in these patients encompasses obtaining both an oropharyngeal and nasopharyngeal swab, since these specimens are those characterized by the highest viral load compared to other biological matrices [12, 13]. Saliva could be considered an alternative option when other samples cannot be collected from symptomatic patients [6, 7, 14].

The Omicron lineages BA.1, BA.1.1 and BA.3 are paradigmatic examples of variants that may generate a specific test failure [15]. The emergence of a 69–70del mutation in these variants causes a 6-nucleotide deletion (21765–21770) in the S gene of SARS-CoV-2, which results in deletion of two amino acids located between the positions 69 (histidine) and 70 (valine) of the spike protein, thus impairing the probe annealing in certain assays, ultimately leading to S-gene target failure (SGTF) [16].

The main characteristics of the molecular tests, as mutated from existing guidelines from other groups are summarized in Table 2. Briefly, at least two SARS-CoV-2 gene targets must always be used by each method, primers should be preferably designed to detect the SARS-CoV-2 E, N, S, ORF1ab and RdRp genes, test positivity must be defined based on manufacturer’s recommended cut-off of viral load and considerations could be made for reporting the viral load (i.e., the cycle threshold (Ct) value), accompanied by name and technical characteristics (i.e., target genes and relative cut-off) of the molecular assay. Reporting the viral load (e.g., the Ct value) may help predicting emergence and progression of SARS-CoV-2 outbreaks, the risk of developing severe COVID-19 illness as well as the SARS-CoV-2 infectiousness [17]. Direct comparison of Ct values obtained with different techniques must be avoided, since the standardization of this measure remains an unresolved matter [18]. Importantly, each assay must be validated against newly emerged SARS-CoV-2

<table>
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<tr>
<th>Setting</th>
<th>Timeline</th>
<th>Test</th>
<th>Ideal sample</th>
<th>TAT</th>
<th>Confirmation</th>
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<tr>
<td>High risk of SARS-CoV-2 infection</td>
<td>ASAP</td>
<td>Lab-based molecular assay</td>
<td>Upper or lower respiratory tract samples. Saliva in specific circumstances</td>
<td>&lt;6–12 h</td>
<td>Not required usually. Repeat with another lab-based molecular assay within 24–48 h when negative in high risk patients</td>
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<tr>
<td>(symptomatic patients)</td>
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<tr>
<td>Low risk of SARS-CoV-2 infection</td>
<td>ASAP</td>
<td>POC-based molecular assay or lab-based</td>
<td>Upper respiratory tract sample. Saliva in specific circumstances</td>
<td>&lt;2 h</td>
<td>Repeat with lab-based molecular assay within 24–48 h in positive patients</td>
</tr>
<tr>
<td>(asymptomatic subjects) at hospital admission/contact tracing</td>
<td></td>
<td>antigen immunoassay</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Asymptomatic subjects</td>
<td>Not recommended</td>
<td>Lab-based, POC-based</td>
<td>Upper respiratory tract sample and/or saliva</td>
<td>1–3 days</td>
<td>Repeat with lab-based molecular assay within 24–48 h in positive patients</td>
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<td>Epidemiologic surveys/population screening</td>
<td></td>
<td>or rapid antigen immunoassays Next generation sequencing (NGS)</td>
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<td>Identification of new</td>
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<tr>
<td>variants</td>
<td>emergence of new lineage(s)</td>
<td></td>
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**Table 1: SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) test indications in specific settings.**

1 Ideally both oropharyngeal and nasopharyngeal swabs; b sputum and/or endotracheal aspirate or bronchoalveolar lavage; c when other samples cannot be collected or for shortage of molecular tests; d signs or symptoms suggestive of SARS-CoV-2 infection and/or suggestive radiological findings. ASAP, as soon as possible; POC, point of care.
variants, to prevent the risk of false negative test results (especially those attributable to gene dropout).

Low risk of SARS-CoV-2 infection (asymptomatic subjects) at hospital admission/contact tracing

The rapid detection of SARS-CoV-2 infection in individual with close contacts with infected people (i.e., a process known as “contact tracing”) and in all patients seeking hospital care for other health conditions but displaying a low clinical risk of having SARS-CoV-2 infection (typically asymptomatic subjects or those without suggestive signs and symptoms of coronavirus disease 2019 (COVID-19)) is important, since this could lead to a more efficient differential diagnosis with other pathologies, as well as to define specific pathways of care for the infected patients, preventing or limiting the risk of emergence of hospital outbreaks [19]. In this specific circumstance, the most important aspect is to safeguard the safety of the hospital staff and of other patients, which actually translates into the possible usage of different diagnostic strategies. Among these, the use of point-of-care (POC) molecular assays has been reported as a valuable surrogate of routine laboratory-based diagnostics, since the diagnostic sensitivity and specificity of several of these tests is only marginally lower (i.e., as high as 95 and 99%, respectively) compared to that of laboratory-based molecular assay [20]. These methods, although plagued by a low throughput (i.e., are typically single-test or few-samples devices), seem especially suited for rapid screening of patients, since test results can be provided in a very short timeframe, thus allowing efficient patient triage. A possible alternative to these methods is represented by the use of laboratory-based antigen immunoassays. These tests, both the so-called “third generation” microfluidic immunoassays [21] and especially the “fourth-generation” laboratory-based chemiluminescent immunoassays, have been developed for use on immunochemistry platforms that are widely available in the vast majority of clinical laboratories, provide rapid test results (typically in less than 1 h) and can hence be used also as stat (urgent) tests. The diagnostic sensitivity of most of these tests is lower compared to laboratory-based molecular techniques (typically ranging between 60 and 90%), though often accompanied by excellent specificity (always around 99%) [22–25]. Their negative predictive value considerably increases in samples with high viral load (i.e., >90% in samples with Ct values <25–30), even with salivary samples [26, 27]. Therefore, these methods could be reliably used for rapid identification of patients with high SARS-CoV-2 viral load, that may remain otherwise undetected and who may be responsible for larger spread of infections within healthcare environments. Due to the risk of obtaining false positive test results with these immunoassays, all patients testing positive should undergo a second laboratory-based molecular test (which has very low risk of false-positive results) within 24–48 h from the first SARS-CoV-2 antigen positivity [28].

As specifically concerns the main characteristics of SARS-CoV-2 antigen immunoassays, as summarized in Table 2, these encompass that the antigen target should be (preferably) the nucleocapsid (N) protein, since this moiety seems less vulnerable to selective pressure to develop mutations and is not subjected to the risk of false positive test results in patients undergoing COVID-19 vaccination with vaccines encoding the spike protein. However, mutations in the N protein have been reported in multiple variants, with Omicron possessing several, including a deletion, which may impact diagnostic assay performance [16, 29]. Antigen immunoassay test positivity must be defined based on manufacturer’s recommended cut-off of antigen concentration, and considerations should be made for reporting the antigen concentration, accompanied by

<table>
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<th>Table 2: Characteristics of SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) tests.</th>
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<td>(a) SARS-CoV-2 molecular assays</td>
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<tr>
<td>– At least two SARS-CoV-2 gene targets must always be tested</td>
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<tr>
<td>– The primers should be preferably designed to detect the SARS-CoV-2 E, N, S, ORF1ab and RdRp genes</td>
</tr>
<tr>
<td>– Each assay must be validated against newly emerged SARS-CoV-2 variants, to prevent the risk of false negative test results</td>
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<tr>
<td>– Test positivity must be defined based on manufacturer’s recommended cut-off of viral load</td>
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<tr>
<td>– Considerations could be made for reporting the viral load (i.e., cycle threshold value), accompanied by name and technical characteristics (i.e., genes and relative cut-off) of the molecular assay</td>
</tr>
<tr>
<td>– Avoid direct comparison of viral load obtained with different molecular assays</td>
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<tr>
<td>(b) SARS-CoV-2 antigen immunoassays</td>
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<tr>
<td>– Antigen immunoassays shall preferably target the SARS-CoV-2 N protein</td>
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<tr>
<td>– Each immunoassay must be validated against newly emerged SARS-CoV-2 variants, to prevent false negative test results</td>
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<tr>
<td>– Test positivity must be defined based on manufacturer’s recommended cut-off of antigen concentration</td>
</tr>
<tr>
<td>– Considerations could be made for reporting the antigen concentration, accompanied by name and technical characteristics (i.e., lower limit of detection) of the assay</td>
</tr>
<tr>
<td>– Avoid direct comparison of viral antigen concentration obtained with different immunoassays</td>
</tr>
<tr>
<td>– Lab-based immunoassays display better diagnostic sensitivity and accuracy compared to rapid manual tests</td>
</tr>
</tbody>
</table>
name and technical characteristics (i.e., lower limit of detection) of the immunoassay. Direct comparison of viral antigen concentration obtained with different techniques must instead be avoided, since the standardization of this measure remains an unresolved matter. As for molecular assays, each immunoassay must also be validated against newly emerged SARS-CoV-2 variants, to prevent false negative test results.

The ideal sample for diagnosing SARS-CoV-2 at hospital admission and/or in close contacts of infected people is an upper respiratory specimen (both oropharyngeal and nasopharyngeal). Saliva could be considered a reliable option for antigen immunoassays, especially in a setting with shortage of laboratory-based molecular tests [6, 7, 14].

**Asymptomatic subjects**

Routine testing of asymptomatic subjects for detecting a potential SARS-CoV-2 infection is not recommended outside specific circumstances such as epidemiologic surveys or population screening, as summarized in the following paragraph. This conclusion is supported by a number of factors, encompassing an ongoing worldwide shortage of supplies and human resources that would be needed for widespread SARS-CoV-2 testing, the significant risk of obtaining false positive test results, the unnecessary isolation of patients with non-evolving illness and/or and scarce infectivity, as well as for physiological distress and health risks that may be caused by over-diagnosis and overtreatment [30].

**Epidemiologic surveys/population screening**

SARS-CoV-2 testing for purposes of epidemiologic surveys and/or contact tracing can also be planned according to a different path, whereby the level of urgency and accuracy needed is understandably lower compared to other circumstances. In this specific case, the timeline is variable, depending on the expected outcome and deliverables of testing (i.e., definition of the size of local outbreaks, monitoring viral spread in certain geographical areas or environmental settings, etc.). The diagnostic resources needed to fulfill these purposes do not require such a high level of accuracy as for other conditions, such that laboratory-based, POC-based or rapid antigen immunoassays could all be used for this scope, depending on the local organization. Notably, the clinical performance of rapid manual antigen immunoassays (i.e., the so-called SARS-CoV-2 antigen rapid detection tests (Ag-RDTs)) is considerably lower than that of other molecular or laboratory-based immunoassay, and could hence be most useful for detecting patients with high viral load (i.e., Ct values <25), whilst their diagnostic sensitivity in those with modest or low viral load is impaired (i.e., around 30–50%) [20, 31], leading to potential false negative results. As in other settings, the significant risk of obtaining false positive test results suggests that all patients testing positive with Ag-RDTs should ideally undergo a second laboratory-based molecular test within 24–48 h from the first positivity [32]. Nonetheless, Ag-RDTs may be useful in reducing the burden on clinical laboratories and providing a more convenient testing method to the public, however, these benefits must be weighed against several limitations, including the previously noted poorer sensitivity leading to potential false negative results with a subsequent lack of isolation of infectious cases, reduced capacity to track the epidemiology of the pandemic, and potential performance issues, especially in context of novel variants. Ag-RDTs should undergo clinical evaluation for diagnostic performance whenever novel variants emerge, along with production of updated guidance on how to use and when to test given the characteristics of that specific variant in the evolving pandemic.

**Identification of new variants**

The application of gene sequencing to SARS-CoV-2 diagnostics has many important clinical implications, including monitoring emerging lineages that may impact human health, investigating the relationship between clades/lineages and epidemiological data, assessing the impact of mutations on performance of molecular/antigenic diagnostic methods, assessing and confirming reinfections, prompting research to assess the impact of mutations in pathogenesis of disease, along with assessing the impact of mutations on therapies and “vaccine escape”. As concerns the possible tools, we suggest that the use of specific RT-PCR kits may be reserved for detecting selected mutations, Sanger sequencing may be used for identifying specific polymorphisms – for example in the sequence of the spike protein or of the receptor binding domain (RBD) – to be linked to variants of concerns, whilst next generation sequencing (NGS) can be used for whole genome sequencing of the virus and thereby for detecting both known and unknown
SARS-CoV-2 variants (Figure 1) [33]. Although a deep description of advantages and limitations of the different molecular techniques cannot be addressed in the present manuscript, it may be useful to summarize here that PCR has a relatively low cost and is a quite fast and widely available technique, but needs to be continuously updated to stay abreast of emergence of new variants. Sanger sequencing is relatively more expensive and slower, but can only identify multiple known mutations on the same gene. Finally, NGS is quite expensive, relatively slow and is not always accessible outside specialized laboratories, but, on the other hand, allows timely detection of new variants, abolishing the need to carry additional investigations for genotyping [33].

Importantly, NGS is now becoming more and more popular in many clinical laboratories for a kaleidoscope of clinical applications, but may have a substantial role also in diagnostics of viral diseases, including SARS-CoV-2, as previously discussed [34]. Recent evidence attests that this technique may allow to accurately and efficiently monitor the evolution and genetic epidemiology of SARS-CoV-2 samples, detecting specific lineage responsible for infection with high relatively high throughput (e.g., thousands of samples at the same time), with turnaround times that can be lowered to 24–48 h [35]. On the other hand, it is important to mention here that direct PCR-based variant analysis of SARS-CoV-2 using specific primers and probes may enable the detection of a limited set of variants of concern for purposes of epidemiological monitoring [36]. Furthermore, PCR genotyping methods have the advantage of returning results in a clinically actionable timeframe. This was important the omicron variant was resistant to all monoclonal therapy except sotrovimab and the BA.2 variant is resistant to sotrovimab [37]. However, unlike whole genome sequencing, this approach would not obviously allow to detect rare variants, new emergent mutations, and requires frequent updating.

Conclusions

Continued SARS-CoV-2 evolution may produce variants with different biological properties and, this, in turn creates some challenges to the laboratory diagnosis of the disease. In particular, Omicron extensive set of mutations is associated with substantial functional and structural differences compared to previous variants [38, 39]. These differences likely contribute to the 50–90% reduction in risk of hospitalization and mortality of Omicron relative to Delta but also lead to decrease the sensitivity of laboratory tests. Therefore, both PCR and antigen-based tests needs to be continuously adapted to better cover and possibly identify new variants or serotypes. This document provides suggestions to better address laboratory testing in different scenarios, including high-risk, low-risk, asymptomatic subjects as well as for population screening and epidemiological surveys. It should be regarded not as the final page of the book, but as a work in progress to update our knowledge and practice.

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