Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Commentary

SARS-CoV-2 diagnostics: Some reflections on current assays

The coronavirus disease-2019 (COVID-19) pandemic is going to profoundly affect numerous aspects of the knowledge, science and practice of public health. Among these, special attention must be paid to the role of the Clinical Microbiology Laboratory, and especially of Clinical Virology Laboratory, which has greatly attracted the interest of health and multifarious stakeholder organizations. Indeed, to date, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) test results have been used to diagnose COVID-19 as well as to, isolate infected patients, and trace their contacts. Hopefully, when an effective therapy or vaccine is available, rapid and reliable testing for SARS-CoV-2 infection, especially in early stages, will help to properly allocate resources and reduce the spread of the virus.

For the above reasons, we as Microbiologists and Virologists would like to briefly comment on this topic and try to provide more precise information with regard to SARS-CoV-2 testing. In our opinion, in research, diagnosis, and, consequently, in the communication of science, the only things that matter are data. Even if they do not always give us unambiguous or definitive answers, it is crucial that they be accrued rigorously, with no bias whatsoever and, if possible, impartially verified.

Since the discovery of the etiological agent, there has been an unprecedented race to satisfy the huge demand for diagnostic tests and inherent reagents, to the remarkable point that the number of tests available to diagnose the SARS-CoV-2 infection, either directly or indirectly as defined below, exceeds all the tests combined for other viruses. At the time of writing, there are 852 commercially available assays globally (of which 378 molecular assays and 443 immunoassays, the other being "inactivation and other diagnostics") (https://www.finddx.org/covid-19/pipeline/). Many of them have received CE-IVD or USA emergency use authorization marks, but due to the emergency, the accuracy of many assays is questionable and do not guarantee consistency among different production lots.

Let us briefly review the types of assays available, addressing some of the key aspects and offering critical comments.

1) Direct assay to detect SARS-CoV-2 (i.e., the detection of the viral genome, so-called molecular assay, until a couple of months ago when assays detecting SARS-CoV-2 antigens became available).

To date, the vast majority of molecular assays to detect SARS-CoV-2 genome have been based on real-time polymerase chain reaction (PCR). This diagnostic tool is inherently sensitive, but positive results may be due to the presence of acting replicating virus or residual viral nucleic acid (i.e., noninfectious virus). Furthermore, because of the inherent huge variability of sampling—nasopharyngeal swabs and respiratory material cannot provide the same amount of genetic material to be tested—molecular assays are qualitative and provide a gross estimate of "viral load" based on the number of amplification cycles necessary for a sample to score positive.

Despite these important limitations, this method is considered the gold of standard to perform a diagnosis of COVID-19.

Basically, a proper assay for virological diagnosis is still unavailable. Indeed:

- Some assays, or even test runs using reagents from renown manufacturers, yield false-negative results. This may be due to inappropriate sample collection, to the extraction/real-time PCR workflow and, finally, to sensitivity of the assays used. The "Test, re-test, and re-test" motto recently commented in Nature Medicine (which has been adopted in most hospitals) (Ramdas et al., 2020) does not solve the problem and in most cases complicates patient management due to increased, sometimes inacceptable, time to diagnosis, amount of material and lability of the template.
- As mentioned, because of the high number of molecular assays and rush time, PCR panels to detect SARS-CoV-2 have been designed to target different genomic regions and this may have an effect on panel sensitivity; importantly, no direct comparison of sensitivity is available, so far and, in most of the cases, diagnostic accuracy, i.e., clinical sensitivity and specificity, have been not addressed. Basically, most laboratories have opted for one method over the others because of availability of reagents, instruments (already in lab or acquired ad hoc), and price.
- Due to the high complexity of diagnostics, molecular testing is currently performed only in well-resourced laboratory systems; countries with fragile health systems without strong laboratory networks lag behind. Even in western countries, swab results have variable times depending on many factors, especially but not exclusively the analytical platform used. Clearly, an improved test production capacity is one of the ways to increase control of the outbreak. This will reduce the possibility of transmission and help maintain functioning societies.
- Processing large numbers of samples within a short period impairs the normal workflow of microbiology and virology testing laboratories, particularly during the development, evaluation, and implementation of new tests. Because of this, microbiology laboratories have had to reroute their priorities and focus on rush and high throughput results rather than on retesting and performing further and insightful analyses.
- In addition, specimen collection, transport, and processing were slowed due to safety requirements, further impairing the workflow of microbiological and virological diagnosis.
- Many papers on the suitability of saliva instead of respiratory secretions have been published, but a rigorous comparison between saliva and nasopharyngeal swab specimens with

respect to sensitivity in detection of SARS-CoV-2 has been only recently documented (Wyllie et al., 2020). Although there is a great hope for the use of this specimen instead of the respiratory swab, further studies are needed to give definite answers.

- To achieve high sensitivity, some of the commercially available assays have been set up to give positivity even at very high threshold cycles (so-called Ct), for instance >40 Ct. Most spurious signals score positive after 35 cycles and, in the absence of other signs of infection or disease, experienced microbiologists and virologists will re-test the sample, possibly with another system and provided that the 2 methods, if available, are equally sensitive before releasing a statement (Falasca et al., 2020). Unfortunately, as mentioned above, most laboratories, even those with little experience with molecular assays, and although there is no recommendation to that effect from regulatory institutions, have begun using these methods without caring or having the predisposition to re-test before claiming that a sample with >40 Ct was bona fide positive.
- The supplying of reagents is not carefully planned. As a result, the ongoing resurgence of virus transmission in Europe, conveys the same problems we were experiencing in the initial emergency phase. The pooling procedure (Cherif et al., 2020) is an interesting approach but awaits further consolidation and, in our opinion, is likely to be impractical in a situation in which most patients, even in the acute phase, have low virus load in the respiratory material and in the current situation, in which the percentage of positive respiratory swabs is going to exceed 10 %.
- As expected (La Marca et al., 2020) and already demonstrated (Liotti et al., 2020) antigen assays have much lower sensitivity than molecular assays. Because of this, most of the antigen assays, which are easy to perform and provide results in less than half an hour are well-suited for point-of-care testing, and for symptomatic patients. Thus, antigen assay have great expectancies and, in some countries, are largely utilized. Nonetheless, at the time of writing, the above drawback cannot be ignored.

Because of the above considerations, further efforts should be made to improve molecular and antigen assays not only to achieve better management of the single patient but also, and probably more importantly, to break the chain of transmission and, hopefully, to guide toward the most appropriate cure, when the latter is firmly established.

2) Indirect assay to detect SARS-CoV-2 infection (so-called serological assay)

Antibody (Ab) responses to SARS-CoV-2 can be detected in nearly all infected individuals 10 to 19 days following the onset of COVID-19 symptoms (Long et al., 2020a). While it is not known how long these Ab responses will be maintained or whether they will provide protection against re-infection, some surveys have pointed out that asymptomatic or low-grade infected patients did not developed Abs at all or that they, even the neutralizing one, decline in strength and number in the weeks and months following infection (Ibarrondo et al., 2020a, 2020b; Long et al., 2020b). The latter finding is not entirely unexpected if one considers that protective immunity to cold-causing coronaviruses is also short-lived (Edridge et al., 2020).
Furthermore:

- Most of the assays must be still validated looking, not only at their analytical sensitivity and specificity, but also at their clinical performance.
- There are a lot of tests with different sensitivity and specificity and, again, their performances have not yet been compared in reliable studies.
- Some instrumental assays detect Abs directed against the viral receptor, S protein, particularly against the domain binding the cellular receptor. These immunoassays provide readouts that, to some extent, parallel the ones obtained with a standard neutralization assay but the relationship is not clear-cut and cannot be replaced. Some sera score positive with the immunoassay and have no neutralizing Abs at all, while others are negative with the instrumental assay but show low neutralizing activity. To this regard, to our opinion, it is incorrect to not consider that the development of antibodies and their specificity depends on the host other than on the type of assay used to measure them.
- As above, access to these tests is complicated by high prices, broken supply chains, and complex ordering systems.
- In some countries, a respiratory swab must be taken from the seropositive to verify his/her contagiousness. Furthermore, the seropositive subject cannot be sure about his/her protection from subsequent infections due to the above-mentioned likelihood of Ab waning over time.
- Furthermore, if the subject is seronegative, he may become positive while “waiting for the result of the test.”
- Last but not least, there are patients who are not (yet) seropositive but harbor the virus. In a recent survey, we demonstrated that about 2% of health care personnel working in homes for the elderly were SARS-CoV-2 genome positive and showed no symptoms whatsoever (Carozzi et al.).

The serological assay supports clinical management only in some specific care settings, as it may help diagnose COVID-19 cases in patients in gray areas, for example, with interstitial pneumonia but negative swabs. It is also useful for identifying asymptomatic infections and detecting circulation of the virus in the population, and is better if repeated at different times. Because IgG and IgM usually appear after the symptoms onset (Long et al., 2020b), serological tests are of little use for early detection of infection.

The reduction and subsequent disappearance of Abs do not necessarily imply that the individual is no longer protected against reinfection or, perhaps, reactivation of an apparently subdued infection, because the immune system may have developed a memory and trigger a prompt and effective response is the individual re-encounters the same antigens. The new frontiers of immunologic assays measuring the cell-mediated response and immunologic memory should provide an answer on this method. Further, if these methods are cheap, easy to use, reliable and, in short, suited for diagnostic purposes and mass studies, they will provide a better estimate of the real prevalence of the infection which, based on rapid IgG/IgM lateral flow assays, appears to be underestimated. Interpretation of antibody tests will be further complicated by the release of SARS-CoV-2 vaccines that, depending on their makeup, could generate an antibody profile indistinguishable from past infection or hyper-reactivity toward the S protein.

Concluding remarks

Laboratory testing plays the principal role in early detection of infected persons, enabling recognition of the infection source and cutting off the transmission route.

Currently, the diagnosis of COVID-19 depends on reverse transcription polymerase chain reaction (RT-PCR) tests directed at the SARS-CoV-2 genome in samples from the nasopharynx or respiratory sites. However, results are affected by various issues, mainly related to clinical specimens (often with a low viral load), to the gene sequence used as a target, and to the technological platform used; furthermore, results may be persistently positive while not indicating infectivity (Kang et al., 2020; Sun et al., 2020a; Carmo et al., 2020).

Preliminary data on real-time PCR Ct value compared with a reduced recovery of SARS Cov2 in cell-culture are becoming available (Bullard et al., 2020; Binnicker, 2020) providing information on the period of virus infectivity. These studies can help confirm that PCR

positivity is likely not the only reliable surrogate marker for determining a patient's infectious status. Recent data (Walsh et al., 2020) suggest that virus infectivity is likely to be highest during the first week of illness and declines between days 8 and 11 post onset of symptoms. Diminution precedes the clearance of the virus that usually occurs within 2 weeks from symptoms onset. In some people, however, the virus can be detected by RT-PCR and isolated in cell culture for up to 50 days in nasopharyngeal swabs and feces (Sun et al., 2020b). The combined application of these 2 techniques should provide a reliable estimate of viral shedding from the various body fluids.

To fully understand the natural history of infection and gain insight on virus-host interplay, we should use a combination of assays to determine the presence and amount of the virus by RT-PCR, expression of viral proteins by antigen detection, and, finally, viral shedding by culture isolation. Furthermore, we will need novel tests to discriminate between reinfection or reactivation of a persisting infection as recently reported (Carozzi et al.). Based on previous viruses, it is likely that these assays will focus on type and strength of immune response (e.g., avidity, immune response against specific antigens) rather than expression of viral genes or other virological parameters.

The virus was discovered only a few months ago and we know very little about it. Therefore, we have to wait for further trials and experimentation, with awareness that good research on virology, virological diagnosis and, in general, biomedicine must be careful, cautious and, when necessary, slow.

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