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SARS-CoV-2 Infection and the COVID-19 Pandemic Emergency: The Importance of Diagnostic Methods

Marco Ciotti[a] Francesca Benedetti[b] Davide Zella[b] Silvia Angeletti[c]
Massimo Ciccozzi[d] Sergio Bernardini[e, f]

[a]Virology Unit, Laboratory of Clinical Microbiology and Virology, Polyclinic Tor Vergata Foundation, Rome, Italy; [b]Department of Biochemistry and Molecular Biology, Institute of Human Virology, University of Maryland School of Medicine, Baltimore, MD, USA; [c]Unit of Clinical Laboratory Science, University Campus Bio-Medico of Rome, Rome, Italy; [d]Unit of Medical Statistics and Molecular Epidemiology, University Campus Bio-Medico of Rome, Rome, Italy; [e]Department of Experimental Medicine, University of Rome Tor Vergata, Rome, Italy; [f]Department of Laboratory Medicine, Polyclinic Tor Vergata Foundation, Rome, Italy

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Abstract
Background: Currently, a pandemic of coronavirus disease 2019 (COVID-19) caused by the novel coronavirus severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is underway, resulting in high morbidity and mortality across the globe. ***Summary:*** A prompt and effective diagnosis is crucial to identify infected individuals, to monitor the infection, to perform contact tracing, and to limit the spread of the virus. Since the announcement of this public health emergency, several diagnostic methods have been developed including molecular and serological assays, and more recently biosensors. Here, we present the use of these assays as well as their main technical features, advantages, and limits. ***Key Messages:*** The development of reliable diagnostic assays is crucial not only for a correct diagnosis and containment of COVID-19 pandemic, but also for the decision-making process that is behind the clinical decisions, eventually contributing to the improvement of patient management. Furthermore, with the advent of vaccine and therapeutic monoclonal antibodies against SARS-CoV-2, serological assays will be instrumental for the validation of these new therapeutic options.

Introduction

An outbreak of unknown pneumonia was reported at the end of December 2019 in Wuhan, Hubei province, China. From the respiratory secretions of affected patients was isolated, a novel coronavirus whose genome analysis indicated belonging to the genus β-coronavirus, lineage B, subgenus *Sarbecovirus*. This seventh human coronavirus is related to some severe acute respiratory syndrome (SARS)-like coronaviruses detected in bats, but it is distinct from the SARS-CoV and MERS-CoV [1]. The new coronavirus

karger@karger.com
www.karger.com/che

Marco Ciotti
Virology Unit, Laboratory of Clinical Microbiology and Virology
Polyclinic Tor Vergata Foundation
Viale Oxford 81, IT–00133 Rome (Italy)
marco.ciotti@ptvonline.it

was named SARS-CoV-2. Since its discovery, SARS-CoV-2 spread all over the world, and at the time of writing >85 million people are infected and almost 2 million died (January 3, 2021). Exposure to the virus may result in asymptomatic infection or development of symptoms that may range from mild upper respiratory tract symptoms to severe pneumonia with respiratory failure, hypercoagulation, hyperinflammatory manifestations, and eventually death because of multiple organ failure [2, 3]. In Italy, according to the report released by Istituto Superiore Sanità updated to December 2, 2020 (https://www.epicentro.iss.it/coronavirus/sars-cov-2-decessi-italia), the mean age of patients dying for SARS-CoV-2 infection is 80 years, most deaths occur in men, and 63.1% of deceased men positive to SARS-CoV-2 suffers from 3 or more comorbidities. Among these, the most common are hypertension (64.2%), type 2 diabetes (30.7%), and ischemic heart disease (30.7%). In order to contain the pandemic, an effective and rapid diagnosis of SARS-CoV-2 suspected infection is required. Several diagnostics methods have been developed in a short time frame since the beginning of coronavirus disease 2019 (COVID-19) pandemic including molecular and serological assays [4], and more recently very innovative methods such as biosensors which are currently under investigation and require validation [5, 6].

Currently, real-time PCR is the gold standard for SARS-CoV-2 testing or for confirming COVID-19 diagnosis. However, it requires skilled personnel and time-consuming laboratory procedures. For this reason, the development of innovative approaches such as biosensors is welcome. They could facilitate the control of outbreaks allowing for a diagnosis of infection at earlier stages thus reducing the rate of transmission, morbidity, and mortality. In this review, we address the use of these diagnostic assays, underlying their advantages and limits, and reporting on their main technical features.

SARS-CoV-2 Diagnostics

Real-Time PCR Assays

Several molecular assays have been validated and are currently available on the market for the diagnosis of SARS-CoV-2 infection. Validated specimen types include nasopharyngeal swab, nasopharyngeal aspirate, oropharyngeal swab, bronchoalveolar lavage, and sputum [7]. More recently, saliva has also been evaluated but awaits validation. Preliminary results indicate saliva as a promising biological specimen for diagnosis, monitoring, and infection control [8].

Suspected SARS-CoV-2 infections are confirmed after detection of unique and specific target regions within the viral genome. According to the PCR design, the specific target regions may include ORF1ab, RdRp, N, and S genes. In addition, some commercial assays include also the amplification of the common beta coronavirus E gene, which is amplified along with one or more specific target genes. In Table 1 are reported the molecular assays approved by the Italian Ministry of Health (0011715-03/04/2020-DGPRE-DGPRE-P).

In the USA, the Centers for Disease Control and Prevention (CDC) developed a real-time PCR protocol, which targets 2 regions of the N gene of SARS-CoV-2. The internal control is represented by the human RNase P gene that is detected both in clinical specimens and control samples (https://www.fda.gov/media/134922/download. Revision 3, March 30, 2020).

Real-time PCR assays represent the gold standard for the laboratory diagnosis of SARS-CoV-2 infection; however, false-negative results may occur. Several factors may be responsible for these incorrect results: quality of the specimen, viral load below the limit of detection (LOD) of the method, incorrect handling of the specimen, problems during shipment, timing of sampling (sample collected too early or too late during infection), and source of sample (upper or lower respiratory tract). In the initial phase of COVID-19 disease, upper respiratory tract sample can result in RT-PCR negative, while chest computed tomography images show the presence of pulmonary abnormalities consistent with viral pneumonia [9–11]. Repeat testing can increase the chance of detecting SARS-CoV-2 RNA [12].

Point of care (POC) molecular tests are designed to deliver results in <1 h using RT-PCR technology and are performed on individuals with suspected COVID-19. They do not require particularly trained personnel; hands-on time is minimal (~1–2 min), and thier result interpretation is straightforward. POC assays may facilitate the management and triage of patients, and some of these platforms could be used outside hospital settings such as in nursing homes to screen the elderly population, which is at high risk of developing pneumonia with consequences often fatal. In Table 2 are listed some of the rapid molecular tests that can be used for qualitative detection of SARS-CoV-2 RNA in individuals with signs and symptoms of suspected COVID-19.

Although correlations have been done between viral load and severity of disease [13], Ct values cannot be used to assess disease's severity or to monitor response to therapy yet. However, low Ct values, indicative of high viral loads, may be used to indicate transmissibility [14, 15]

Table 1. Real-time reverse transcription PCR assays authorized by the Italian Ministry of Health

Assay	Specimen type	Gene target	LOD	Manufacturer
Bosphore Novel Coronavirus (2019-Ncov) detection kit	Nasopharyngeal swab, oropharyngeal swab, sputum, bronchoalveolar lavage	E, orf1ab	25 copies/reaction	Anatolia Genetik, Turkey
STANDARD M nCoV Real-Time Detection Kit	Nasopharyngeal swab and throat swab, sputum	E, ORF1ab	NA*	SD BIOSENSOR Inc, Korea
Allplex™ SARS-CoV-2 assay	Sputum, nasopharyngeal swab, nasopharyngeal aspirate, bronchoalveolar lavage, throat swab	E, RdRp, N, S	50 copies/reaction	Seegene Inc., Korea
QUANTY COVID-19	Nasopharyngeal swab, oropharyngeal swab, sputum, serum	N	NA*	CLONIT SRL, Italy
GENEFINDER COVID-19 PLUS REALAMP KIT	Bronchoalveolar lavage fluid, throat swab, sputum	E, RdRp, N	10 copies/reaction	OSANG HEALTHCARE Co., Korea
Novel Coronavirus COVID-19 (2019 nCoV) Real Time Multiplex RT PCR Kit	Nasopharyngeal swab, oropharyngeal swab, bronchoalveolar lavage, sputum, endotracheal aspirate	E, N, ORF1ab	1×10^3 copies/mL	Liferiver, SHANGHAI ZI BIO-TECH CO., LTD, China
LabGunTM COVID-19 RT-PCR Kit	Nasopharyngeal swab, oropharyngeal swab, nasopharyngeal wash/aspirate, nasal aspirate, sputum	E, RdRp	20 copies/μL	LabGenomics Co., Ltd, Korea
REALQUALITY RQ-2019-nCoV	Nasopharyngeal swab, bronchoalveolar lavage fluid, sputum	E, RdRp	NA*	AB ANALITICA s.r.l., Italy
COVID-19 detection kit	Nasopharyngeal swab, oropharyngeal swab, brongho-alveolar lavage	N, ORF1ab	500 copies/mL	OACP S.R.L., Italy

* NA, not available; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; LOD, limit of detection.

Table 2. Rapid molecular tests for detection of SARS-CoV-2 RNA in respiratory samples

Molecular assay	Specimen type	Gene target	LOD	Time to results, min	Company
Xpert Xpress SARS-CoV-2	Nasopharyngeal, nasal, mid-turbinate swab	E, N2	250 copies/mL	45	Cepheid, Sunnyvale, CA, USA
QIAstat-Dx respiratory SARS-CoV-2 panel	Nasopharyngeal swab	E, ORF1b,RdRp	500 copies/mL	60	Qiagen, Hilden, Germany
BIOFIRE® respiratory panel 2.1	Nasopharyngeal swab	S, M	160 copies/mL	45	bioMérieux, Marcy l'Etoile, France
Simplexa™ COVID-19 direct kit	Nasal swab, nasopharyngeal swab, nasal wash/aspirate, and bronchoalveolar lavage	ORF1ab, S	500 copies/mL (NPS, NW/A); 242 copies/mL (NS): 1,208 copies/mL (BAL)	60	DiaSorin Molecular LLC, Cypress, CA, USA
VitaPCR™ SARS-CoV-2 assay	Nasopharyngeal swab, oropharyngeal swab	N	2.73 copies/μL	20	Menarini Diagnostics, Florence, Italy
ID NOW COVID-19 assay	Nasopharyngeal swab, throat swab	RdRp	125 genome equivalents/mL	13	Abbott Diagnostics, IL, USA

COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; LOD, limit of detection.

SHERLOCK One-Pot and DETECTR Testing

SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) assay combines simplified viral RNA extraction with isothermal amplification and CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-mediated detection. The assay was designed to detect the N gene of SARS-CoV-2 by optimized sets of LAMP (Loop-mediated isothermal AMPlification) primers and AapCas12b guide RNA. The assay is highly sensitive (33 copies/mL vs. 1,000 copies/mL of the CDC qRT-PCR), and the results can be read on a lateral-flow strip or fluorescence reader after 80 or 45 min, respectively [16]. Similarly, DETECRT (SARS-CoV-2 DNA Endonuclease-TargEted CRISPR Trans Reporter) assay performs reverse transcription and isothermal amplification of RNA extracted from nasopharyngeal or oropharyngeal swabs, followed by Cas12-based detection of E and N gene sequences. The N sequence targeted by DETECTR differs from that recognized by the CDC assay (N1 and N3 regions). The selected Cas12 guide RNAs allows the identification of SARS-CoV-2, bat SARS-like coronavirus and SARS-CoV in the E gene, whereas the N region is specific for SARS-CoV-2. The test is positive if both the E and N genes are detected or presumptive positive if either E or N gene is detected. The LOD of the assay is 10 copies/μL reaction versus 1 copy/μL of the CDC assay. The positive predictive agreement and negative predictive agreement of DETECTR assay versus CDC qRT-PCR are 95 and 100%, respectively. The turn-around time of the DETECTR assay from extraction to result is about 52 min for 1–8 samples extracted manually. Results are visualized by a fluorescent reader or lateral flow strip [17].

Serological Assays

Serological assays have been developed using as target the highly antigenic structural proteins spike (S) and nucleocapsid (N) of SARS-CoV-2. In most individuals, measurable antibodies develop within days or weeks from symptoms onset [18, 19], thus limiting the use of serological testing in the early phase of infection [20, 21]. However, serological assays are important for contact tracing, for identifying suspected cases who are PCR negative but show radiological findings suggestive of COVID-19 [9, 10], to identify asymptomatic carriers [22] or to determine the development of neutralizing antibodies in response to vaccination [23, 24]. Instead, doubts arise about the use of antibody tests for seroprevalence surveys for public health management reasons because the duration of circulating antibody is currently unknown [25].

Most of the available serological assays detect IgM and IgG antibodies although IgA antibodies play an important role in mucosal immunity. IgAs can be detected earlier than IgGs, and in atypical cases or in patients with repeated negative RT-PCR, IgA along with IgG may contribute to the diagnosis of SARS-CoV-2 infection [26].

Several commercial serological assays have been developed since the beginning of the COVID-19 pandemic such as ELISAs, chemiluminescence assays (CLIAs), and lateral flow assays. Because of the differences in the assay design, immunoglobulin classes detected (IgM, IgG, and IgA), SARS-CoV-2 antigen used (receptor binding domain [RBD], S protein, S1 subunit, N protein) or native inactivated SARS-CoV-2 [27], and specimen type (serum, plasma, whole blood, and finger-stick whole blood) results are often not comparable. Furthermore, it is important to verify the performance characteristics of the assays through a validation process that takes into account the analytical features of the assay as well as the clinical sensitivity of the test results [28].

Serological assays can be qualitative or semiquantitative and show different sensitivity and specificity toward the antibodies detected. In a recent study where was compared the performance of ELISA, CLIA, and ECLIA assays, it was found that overall, the ECLIA assay performed best showing an optimal sensitivity and specificity since the first days of infection suggesting that it could be considered a valid screening method. IgAs were detected earlier than IgMs by ELISA assay suggesting that IgA detection can be more useful than IgM in the early diagnosis of SARS-CoV-2 infection. Also, its level was higher than IgM over 20 days of observation [29]. A similar observation was already reported in literature [30]. Instead, IgGs were consistently detected after 10 days from symptoms onset in line with other studies [29, 31].

Magnetic chemiluminescence enzyme immunoassay is a double antibody sandwich immunoassay that uses the nucleoprotein and a peptide of the spike protein of SARS-CoV-2 as recombinant antigens for the detection of IgM and IgG against SARS-CoV-2. Using this assay, IgM and IgG were detected since the first days after symptoms onset, and by 17–19 days from symptoms onset, 100% of the study subjects were IgG positive. The subject's IgM positive reached a peak of 94.1% after 20–22 days from symptoms onset. The median day of seroconversion for both IgM and IgG was 13 days post symptoms onset. Three types of seroconversion were observed: (i) synchronous seroconversion of IgM and IgG; (ii) IgM seroconversion

earlier than IgG; and (iii) IgG seroconversion earlier than IgM. In some patients, IgM and IgG plateaued 6 days after the first positive determination. Furthermore, testing of the sera of individuals with negative RT-PCR and no symptoms who came in close contacts with COVID-19 patients showed that some of these individuals later tested IgM and/or IgG positive. These last cases demonstrate the importance of serology in identifying those suspected cases that are missed by molecular tests [32].

With the introduction of vaccination against SARS-CoV-2, it will be important to monitor the development of neutralizing antibodies against the virus and their duration. At the time of writing, the vaccine Comirnaty developed by Pfizer-BioNTech against SARS-CoV-2 has received both the Food and Drug Administration and European Medicine Agency approval (Pfizer-BioNTech COVID-19 Vaccine|FDA; European Medicine Agency recommends first COVID-19 vaccine for authorization in the EU|European Medicines Agency [europa.eu]), and the approval for the AIFA (Agenzia Italiana del Farmaco), and is being distributed in Italy. This vaccine demonstrated a 95% efficacy in clinical trial in people 16 years of age or older [33].

Recently, an immunoassay, Elecsys® Anti-SARS-CoV-2 S (Roche Diagnostics, Monza, Italy), has been released which is designed for the quantitative determination of antibodies to the SARS-CoV-2 spike RBD in human serum and plasma. The assay uses a recombinant RBD of the S antigen in a double-antigen sandwich assay format that favors detection of high-affinity antibodies against SARS-CoV-2. The assay aims at evaluating the adaptive humoral immune response to the S protein of SARS-CoV-2 [34]. This assay showed a clinical sensitivity of 98.8% (95% CI: 98.1–99.3%) when testing samples collected 14 days or later after confirmed SARS-CoV-2 diagnosis by PCR, and an analytical specificity of 100% (95% CI: 99.7–100%) when testing samples collected before October 2019, including samples from individuals with common cold symptoms, samples from individuals infected with 1 of the 4 common cold coronaviruses (HKU1, NL63, 229E, or OC43), and anti-MERS-CoV positive samples. The clinical specificity was 99.98% (95% CI: 99.91–100%), when testing pre-pandemic samples obtained from routine diagnostics and blood donors (Roche Diagnostics International Ltd, Rotkreuz, Switzerland).

Rapid Diagnostic Assays

There are 2 types of rapid diagnostic assays: the antigen detection assay and the antibody detection assay. Both tests can be used at the POC or near it and do not require special equipment or laboratory infrastructures. They are particularly useful in settings where more expensive equipment or reagents are not available or when it is necessary to reduce the pressure on the molecular biology laboratories [35].

The antigen detection assay is usually directed against the nucleocapsid protein of the SARS-CoV-2 which is produced during active infection. The assay is performed on nasopharyngeal swab samples that are released into a dedicated or universal transport medium. The result of the antigen detection assay may be influenced by several factors including the time of sample collection, the quality of sampling, the concentration of the virus in the collected sample, and the quality of the reagents. The sensitivity of the assay is low compared to molecular tests [36], and it is generally positive when the viral load is very high and the subject is very infectious, that is, in the first days of infection during the asymptomatic phase (1–2 days) and few days after symptoms onset (5–7 days). The antigen test is usually negative when the Ct value of the real-time PCR is over 30, while its sensitivity increases with Ct values below 25 [36]. Therefore, a negative antigen test result cannot exclude a SARS-CoV-2 infection, and cannot be used to provide guidance for quarantine decision. Nonetheless, rapid antigen tests may be useful in high prevalence settings, where a positive result most likely represents a true positive result or in the presence of asymptomatic carriers with high viral load where accelerates contact tracing [37]. Time to result is about 15 min.

Rapid antibody tests are easy to perform and can be used at the POC returning a result within 15–20 min. The test can be run on whole blood, finger-stick whole blood, serum, and plasma. Rapid antibody tests can be used to detect previous SARS-CoV-2 infection and should not be used for determining active infections in clinical care or for contact-tracing purposes. A positive result does not necessarily reflect the presence of neutralizing antibodies or protective immunity [38].

Rapid antibody tests are not useful for the early diagnosis of SARS-CoV-2 infection. Overall, antibody tests have an accuracy of 30% in the first week after symptoms onset, that increases to 70% in the second week, and to >90% after 3 weeks [25]. Sensitivities, specificity, and accuracy vary with the manufacturer. For instance, in a study performed in our laboratory, rapid tests were compared to CLIA assay. The following results were obtained: CLIA assay showed a sensitivity of 95% for IgG versus about 90% for the immunochromatographic tests, whereas the sensitivity for IgM was 91% for CLIA and ranged

from 61.4 to 87.8% for the rapid tests. Specificity was 100% for all tests [39]. Therefore, these tests should undergo laboratory validation before use in outpatient clinics or as direct-to-consumer testing.

Biosensors in the Diagnosis of SARS-CoV-2 Infection

A rapid and accurate, easy to use, diagnostic system is important for controlling infection source and monitoring progression of disease. Recently, it has been developed an ultrasensitive electrochemical detection technology that uses calixarene functionalized graphene oxide for targeting SARS-CoV-2 RNA. Using a portable electrochemical smartphone, this technology can detect viral RNA without performing reverse transcription and amplification, but instead, it relies on a supersandwich-type recognition approach. The biosensor detected viral RNA from COVID-19 confirmed patients and recovery patients, and the detectable ratio was higher than RT-PCR suggesting a higher sensitivity. In this work, the authors reached a LOD of 200 copies/mL which is much lower than the LODs claimed by several commercial diagnostic RT-PCR assays. Given the high sensitivity and easy to use, the biosensor could be used as a point-of-care (POC) testing [5].

Another interesting approach is the use of a breath device for differentiating between COVID-19 patients and patients with other lung infections. The device is composed of a nanomaterial-based hybrid sensor array able to detect disease-specific biomarkers from exhaled breath [6]. In the case of SARS-CoV-2 infection, this biosensor showed a good capability of differentiating between COVID-19 patients, healthy controls, and patients with lung infections unrelated to COVID-19. Training and test set data showed 94 and 76% accuracy in differentiating patients from controls, and 90 and 95% accuracy in differentiating between COVID-19 patients and patients with other lung infections, respectively [6]. If the validity of this technology is confirmed by future studies, this biosensor may be used as screening tool in POC facilities.

Conclusions

Laboratory diagnostic assays are key for a proper management of COVID-19 patients and for limiting the spread of SARS-CoV-2. Molecular assays represent the gold standard for the diagnosis of suspected COVID-19 cases. Nonetheless, serological assays may be used in combination with molecular tests to improve diagnostic sensitivity and to identify asymptomatic individuals who tested PCR negative but are IgG or IgM positive; in particular close contacts of COVID-19 patients. With the introduction of the prophylactic vaccine against SARS-CoV-2, detection and quantification of neutralizing antibodies by serological assays will allow to verify the efficacy of the vaccine through the determination of the antibody response against the virus and to monitor the neutralizing antibody titer. Monitoring of the antibody titer is important to determine when a new dose of vaccine is needed. Finally, a new impulse in support of the battle against COVID-19 pandemic comes from new technologies such as biosensors that, if validated, might be used in POC facilities.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Marco Ciotti: conception of the work, writing, revision, and final approval; Francesca Benedetti: PubMed search, revision, and final approval; Davide Zella: critical revision and final approval; Silvia Angeletti: critical revision and final approval; Massimo Ciccozzi: critical revision and final approval; Sergio Bernardini: conception of the work, critical revision, and final approval.

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