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Review Article

Updates on Laboratory Diagnosis of COVID-19 infections

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Abstract

Background: COVID-19, the disease caused by SARS-CoV-2, beginning in the late 2019 as an outbreak in Wuhan, China- now, became the world's most feared pandemic among communities around the globe. While we are investing almost all our efforts to save us from the infection, the diagnosis, still at the end of about 2-years of dreadful pandemic, remains difficult for common people. Although, this is a notorious, but enveloped virus, and therefore, is easily destroyable with heat (so, cannot easily survive in the environment) and detergents (so, can be easily killed by applying soap and water), is now returning consecutively in waves of variants with increasing virulence and transmissibility. Because of new variants of the virus, diagnostic approaches to identify the virion as a whole (by cell culture) or in parts (by detecting antigens) or viral products (by detecting antibodies against significant antigens) becomes difficult. The currently available laboratory test methods are ranging from rapid tests at point-of-care (detecting viral antigen(s)) to the genome sequencing. In between, majority of the tests like electron microscopy and cell culture are not routinely practiced, because of their high-end costly equipment and set up. All of these tests have extensively different results due to unpredictably variable presence of the virus (and its products) in clinical specimens as well as costs among the brands. And these limitations are now putting the healthcare professionals along with their patients in discomforts of unsatisfactory management. Yet, the rapid tests are widely practiced for screening purposes, followed by confirmation by real-time reverse transcriptase polymerase chain reaction (rtRT-PCR) tests. This is a simple and comprehensive review of the diagnostic approaches considering all relevant issues of the virus (SARS-CoV-2) and the disease (COVID-19) immunobiology with the publications available up to February, 2022.

Keywords: Acromion process, COVID-19, SARS-CoV-2, Laboratery Diagnosis Update

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Introduction

The real horror name COVID-19 warrants back to the dateline of 31 December 2019, when Wuhan Municipal Health Commission in China reported to the WHO country office that a series of Pneumonia cases emerged in Wuhan (under the province Hubei of China) with clinical presentations resembling viral pneumonia. The cases of the primary outbreaks were mostly found having epidemiological link with the large sea-food market in Wuhan.[1-3] Specimens from the hospitalized patients (majority of them were sellers of the seafood market in Wuhan) were sent to Wuhan Institute of Virology, scientists analysed one of the specimens by metagenomics analysis and found 79.6% sequence identity to SARS-CoV BJ01 (GenBank accession number AY 278488.2). Scientists also found a short region of RNA-dependent RNA polymerase from a bat coronavirus and conducted a full-length sequencing that shows a 96.2% sequence homology.[4,5] Inoculation of respiratory secretions from infected individuals into Vero E6 and Huf7 cell lines and human airway epithelial cells brought to the isolation of a novel virus, whose genome sequence showed belonging to the Coronaviridae family. Soon the virus was characterized as a novel beta coronavirus and named as the '2019-nCoV'.[1,6] The viral infection was found to spread to the surrounding countries very soon potentiating a pandemic threat and then throughout the globe establishing the world's dreadful pandemic.[7] On 30 January, WHO was very scare to declare a Public Health Emergency of International Concern (PHEIC).[7,8] The virus was later renamed by the international Committee on Taxonomy of Viruses (ICTV) as SARS-CoV-2.[8-10]

Although in the last twenty years, mankind has faced three different coronavirus outbreaks (SARS-C0V-1 in 2003,[11] MERS-CoV in 2012,[12] and SARS-CoV-2 pandemic in 2019), the last one appeared as the most devastating in all considerations.[13]

Now, at the end of about two years of dreadful massacres of the world's economy and all-stage livelihoods of the citizens

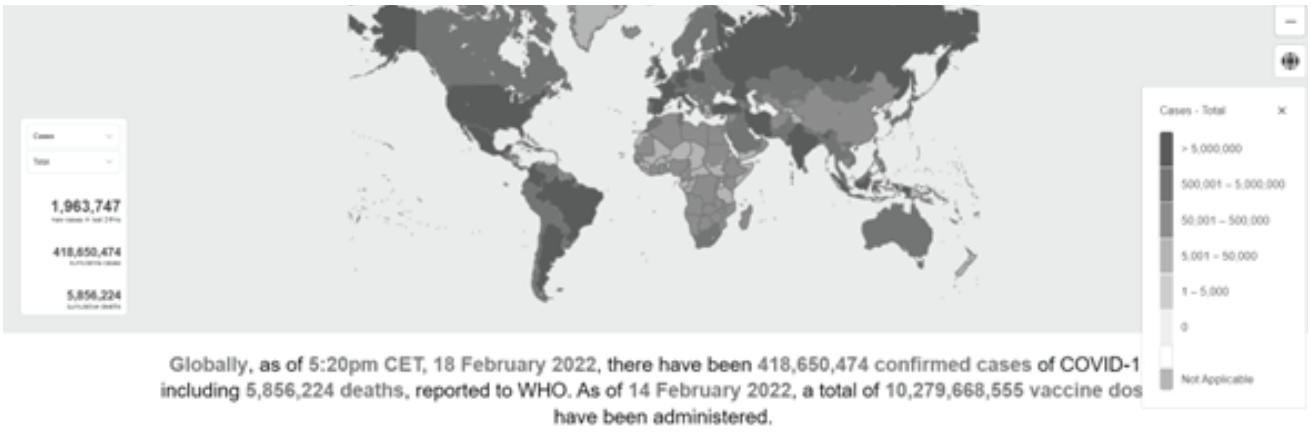

Figure 1: COVID-19 situation as on 18 February, 2022 (source: WHO Coronavirus Dashboard, available at: https://covid19.who.int/ , accessed 20.02.2022)

and availability of many promising vaccines, the transmissions of the virus and consequent morbidity and mortality could not be yet made under good control.[14] (Figure 1).

For laboratory diagnosis of a viral infection, including the SARS-CoV-2 infections, usually multiple evidences are required starting from clinical presentations by the infected person to laboratory data exploring from the clinical specimens. Clinical presentations by the patients are due to the induced pathology upon cells or tissues of the host, culminating into tissue injury and the resultant clinical features experienced. Whereas, laboratory data mostly related to direct (the microorganism itself as observed by microscopic examination, or structural component of the organism as exemplified by detection of antigens or Nucleic acids by molecular diagnostics like Nucleic Acid Amplification Test (NAAT)) or indirect (by detection of antibodies produced in response to the antigens of the organism) evidences in favour of the suspect pathogen.

Brief immunobiology of SARS-CoV-2

While considering laboratory diagnosis of COVID-19, it directs to the identification of SARS-CoV-2, the causative virus, as a whole virion or its structural components like antigens or whole-genome nucleic acid or specific gene segments available in clinical specimens. Therefore, detailed knowledge on structural components and immunobiology of the virus including pathogenesis and evolution of the variants in essential to select right specimen along with the most appropriate test and laboratory preparedness.

As it is known that the SARS-CoV-2 was first documented by the CDC country office China as a novel coronavirus, and is an RNA virus containing approximately 27-32 kb of positive sense single stranded RNA.[9,10,15] This betacoronavirus is an enveloped virus, containing a large nucleoprotein (N) having three trans-membrane protein antigens (S) incorporated into the lipid envelope and two smaller proteins- membrane protein (M) and envelope protein (E).[16-18] When observed under an electron microscope, the virus appears as spherical particle with variable diameters around 100nm without spikes.[19,20] The genome of the SARS-CoV-2 has at least six open reading frames (ORFs) and accessory genes, comprising

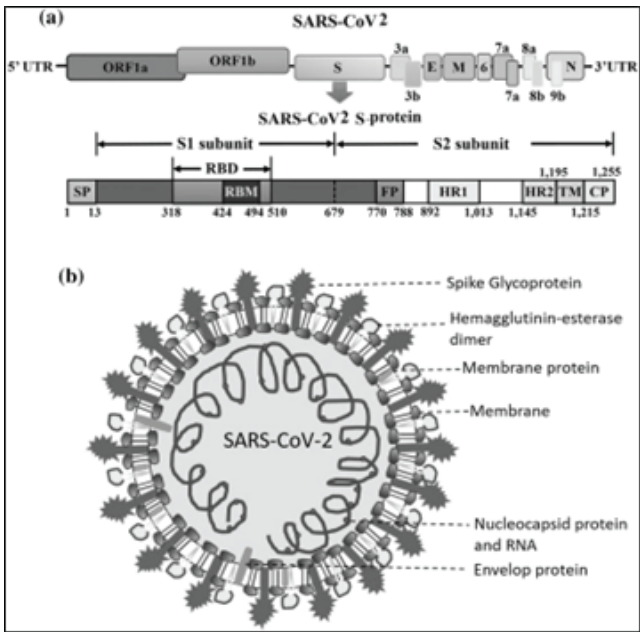

Figure 2: Schematic representation of (a) the genome and (b) virion structure of SARS-CoV-2.
[Reproduced with permission from original source available at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7196923/]

of 11 coding regions that encode 12 potential gene products. At 5′ terminal, two-thirds of the genome consists of two ORFs (e.g., ORF1 and ORF2), which encode two polyproteins namely pp1a and pp1ab which are further cleaved into 11 and 16 non-structural proteins, respectively. The 16 proteins are responsible for genome and viral replication. Whereas, at the 3′ terminal, genes for the structural proteins (e.g., S, E, M & N) are located. Other gene products include spike (S), ORF3a, Envelope (E), Membrane (M), ORF6, ORF7a, ORF7b, ORF8, Nucleocapsid (N), and ORF.[10,15,16,21] Four of these structural proteins are important for coronavirus infectivity, namely S, E, M and N.[13,15,16,21-23] The S protein is responsible for host specificity, viral attachment to the receptor and fusion with cell membrane.[13,22] The N protein interacts with viral RNA to form the ribonucleoprotein and protects viral RNA genome.[23,24] The E protein helps in virion assembly and ion channel actions.[14-16,18,19] The M protein is the key for assembly of viral particles by interacting with all other structural proteins.[13,22-26]

Investigators made it clear that like SARS-CoV-1, SARS-CoV-2 also infects humans through the angiotensin-converting enzyme (ACE-2) which is highly expressed in organs of the humans including respiratory and gastrointestinal tracts, blood vessels, bone marrow, spleen, thymus, lymph node, liver, kidney and brain. This receptor regulates the interspecies and human-to-human transmission through interactions with S protein of the virus.

Soon after the first wave of COVID-19 pandemic during early 2020, most of the areas of the globe face the second and subsequent waves of infection. Scientists could identify the strains of SARS-CoV-2 in the second wave by whole genome sequencing. The new strain in Houston, TX, USA were found to have a Gly614 amino acid replacement in the spike protein- these mutated variants have been found linked to increased transmission and infectivity. Patients infected with the Gly614 variant strains had significantly higher virus loads in the nasopharynx on initial diagnosis.[27]

While the world communities were facing repeated waves of infections and the life-threatening complications due to COVID-19, the globe is now under newer challenges of emerging variants of the SARS-CoV-2. The World Health Organisation (WHO) readily announced the simple, easy-to-say labels for SARS-CoV-2 variants of concern and variants of interest using letters of the Greek alphabet.[28] The variant lineages were also named using computational tool for Phylogenetic Assignment of Named Global Outbreak Lineages (PANGOLin/ PANGO Lineage).[29] Among the variants those having clear evidence indicative of a significant impact on transmissibility and severe morbidity have been identified as Variants of Concern (VOCs) and those having evidences that could imply a significant impact on transmissibility and severity have been identified as Variants of interest (VOIs). Some other variants with genetic changes and having no evidence of phenotypic or epidemiologic impact are suspected of posing a future threat are designated as 'Variants under monitoring (VUM)'.[30-32] Yet another group that have been reclassified on at least one of the following criteria: (i) the variant is no longer circulating, (ii) the variant has been circulating for a long time without any impact on epidemiological situation, (iii) scientific evidence demonstrates that the variant is not associated with any concerning properties, have been designated as 'Formerly Monitored/ De-escalated Variants'.[30,31]

Approach to diagnose COVID-19

Testing strategies for SARS-CoV-2

Selection of tests for SARS-CoV-2 in symptomatic or asymptomatic individuals depends upon the objective of screening, diagnosis or public health surveillance.[33]

Screening tests: are intended to identify people with SARS-CoV-2 infection who are asymptomatic and do not have known or suspected exposure to COVID-19 patients for the purpose of employment/work, travel and study. Screening test helps to identify the unknown cases, so that preventive measures can be taken for further transmission during the individual's stay or movement.[33]

Diagnostic tests: are intended to identify current infection and should be performed on any one who have signs and symptoms consistent with SARS-CoV-2 infection and/or following recent or suspected exposure to COVID-19 patients, irrespective of vaccination status of the individual.[33]

Public health surveillance tests: are intended as a part of the ongoing, systematic collection, analysis and interpretation of health-related data for planning, implementation and evaluation of the intervention measures to control and contain COVID-19 in a community.

Specimen collection

Before planning for the diagnostic approach for COVID-19 infection, appropriate selection of the specimen that will be analysed for the evidence is very important. Because, right selection as well as rightly collection of the appropriate specimen may yield the highest sensitivity of the diagnostic approach. At the same time, adequate safety measures (personal protective equipment (PPE) and packaging for transport) are very crucial for infection prevention and control perspective to break the chain of infection.[34,35]

Upper respiratory tract specimens, including nasopharyngeal swab (NPS) are usually collected. When collection of NPS is not possible, other upper respiratory specimens like oropharyngeal swab, nasal mid-turbinate swab, nasal swab from anterior nares, and nasopharyngeal wash/ aspirate can be collected.[36] In some instances, when infection spreads downwards to involve the lung parenchyma, the virus can be missed- in these cases, lower respiratory tract specimens like sputum or bronchoalveolar lavage fluid (BALF) may be the alternative choice.[37] For initial diagnostic testing, the Centers for Disease Control and Prevention (CDC), USA recommends collection and testing of upper respiratory

specimen.[38] In a study by Yang et al analysing more than 3.5k clinical specimens found that during the first 14 days of symptoms onset (dso), sputum possessed the highest positive yield (73.4%-87.5%), followed by nasal swabs (53.1%-85.3%) for both severe and mild cases of COVID-19.[39] The investigators could identify viral RNA from BALF collected from severe cases within 14 dso and lasted up to 45 days- notably, no viral RNA was identified in BALF from the mild cases. In another study, investigators compared throat washings, nasopharyngeal and oropharyngeal swabs among hospitalized and confirmed COVID-19 patients between 0-15 dso and found good sensitivities of 85%, 85% and 79% respectively.[40] Whereas, another study by Jeong et al demonstrated viable SARS-CoV-2 in saliva, urine and stool specimens of COVID-19 patients up to 11-15 dso. They also demonstrated that viral shedding in saliva, urine and stool specimens were almost equal to or higher than those in nasal/oropharyngeal swabs.[41] Viable viruses have also been isolated from urine and stool specimens from COVID-19 patients up to 11-15 days of clinical course.[41,42] Stool specimens were found excreting the viruses among patients who did not have diarrhoea.[43] In a rapid review, Zhou and O'Leary identified that assessing against a composite standard, anterior nares swabs are less sensitive (42-94%) than nasophayngeal swabs (79-100%).[44] Another systematic review and meta-analysis found sensitivity of saliva (88%) superior to nasal (82%) and oropharyngeal (84%) swabs.[45] And some investigators found saliva comparable to nasopharyngeal swab.[46]

After collection, all specimens for antigen detection should be placed in a tube containing viral transport medium (VTM) and transported to the laboratory as early as possible.[38]

For serological tests, blood is the specimen for detection of antibodies against the virus, including the vaccine effectiveness evaluation- although, some leading authorities including US FDA (Food and Drug Administration) do not recommend antibody testing to assess immune status after vaccination.[47] Antibody detecting tests are especially important among the asymptomatic individuals.

Specimens should be stored at 2-8^{O}C for up to 72 hours after collection- for further delays in shipment or testing, the specimens should be stored at -70^{O}C or below. For transport, specimens should be packed following triple package system for transport of infectious biological specimens.[34,38, 50]

Infection prevention and control (IPC) measures:

Although IPC measures are very essential for all aspects of COVID-19 patient management, including the safety of the patients and healthy people in the community as well as the healthcare workers and the environment, the recommended

Table 1: Specimens for diagnosis of COVID-19 infection

Sl no	Specimen*	Indication	Test(s) name	Sensitivity	Highest Sensitivity on dso	Reference (s)
1.	Nasopharyngeal swab	Screening, Diagnosis & Surveillance (suspected case of COVID-19 at OPD collected by a trained HCP)	NAAT, Ag-detecting RDT	53.1-85.3%	5-7	38,39
2.	Oropharyngeal swab	Diagnosis (suspected case of COVID-19 at OPD collected by a trained HCP)	NAAT	45.7-72.7%	5-7	38,39
3.	Nasal swab from anterior nares/ mid-turbinate swab	Screening & Diagnosis (suspected case of COVID-19 at home or OPD)	Ag-detecting RDT, NAAT	42-94%	5-7	38,43
4.	Nasal/ pharyngeal wash	Diagnosis (suspected case of COVID-19 at home or OPD)	NAAT	85%	5-7	38,39
5.	Saliva	Diagnosis (suspected case of COVID-19 at home or OPD)	NAAT	88%	5-7	38,44
6.	Sputum	Diagnosis (hospitalized cases with respiratory distress)	NAAT	73.4-84.5%	7-14	38,39
7.	Bronchoalveolar lavage fluid	Diagnosis (hospitalized cases with respiratory distress)	NAAT	100%	10-15	38,39
8.	Anal swab/ Faeces	Screening & Surveillance (convalescence period)	NAAT	36.7%	11-15	39, 41-43
9.	Urine	Screening & Surveillance (convalescence period)	NAAT	0%-NA	11-15	39, 41
10.	Blood	Screening & Surveillance (asymptomatic suspects)	Serology (ELISA)	84.3%	14-30	38, 48, 49

Dso- day after symptom onset, NA-not available, *Multiple specimens are recommended for greater sensitivity.

practices of IPC are equally important during laboratory handling of the patient for specimen collection, specimen preparations for testing and disposal. There are very specific recommendations for PPE use and disposal, specimen collection, handling, transport, testing and disposal by the global leading authorities of healthcare system.[34,35,38]

The SARS-CoV-2 is considered as a biosafety level-3 organism. All laboratories handling clinical specimens should perform risk assessments and follow standard precautions, including hand hygiene and use of specific PPE such as laboratory coat or gown, gloves, eye protection or a disposable mask and face shield to protect skin and mucous membrane of the eyes, nose and mouth.[34]

Work surfaces and equipment should be decontaminated using recommended disinfectants like hypochlorite solution, 70-90% Ethanol, povidone-iodine etc.[34,35]

Initial processing of specimens (before inactivation of viruses) should be performed in a properly validated biological safety cabinet (BSC) or an equivalent containment device. Non-propagative laboratory works like NAATs should be conducted in an environment equivalent to biosafety level-2 (BSL-2), whereas, propagative works like virus culture requires a containment laboratory with inward directional airflow equivalent to BSL-3. Point-of-care assays and antigen-detecting rapid diagnostic tests (RDTs) can be performed on a laboratory bench, wearing proper PPE and using appropriate disposal systems in place.[35]

Selection of laboratory test method

For the diagnostic approaches of COVID-19 infection considering sensitivity, specificity and current practices, four common panels of laboratory tests are considered: (i) molecular diagnostics- using the gene sequences of the virus that expresses different proteins of the virion (Nucleic Acid Amplification Tests (NAATs)), or the total genome sequencing for characterization to be useful for developing diagnostic approaches as well the vaccines; (ii) serological tests- the antigens and antibodies of the virus can be identified using corresponding antibody and antigen containing reagents that also includes the rapid tests at point-of-care (POC); (iii) microscopy- the virion (SARS-CoV-2) morphology can be identified by an electron microscope; and (iv) culture- the virus can be cultured in different cell lines including simian and human cells.

In the context of SARS-CoV-2, the newer concepts of laboratory diagnosis that include better antibody reagents and more sensitive assays for direct analysis of specimens, molecular genetics techniques and genomic sequencing for direct identification of the virus should be adopted primarily.

(i) Molecular diagnostics:

Molecular diagnostics in this section, actually refers to nucleic acid-based tests. Currently, nucleic acid amplification tests (NAATs) are the mainstay of confirmatory diagnosis of COVID-19. The NAATs detect nucleic acid (RNA) of SARS-CoV-2, usually from upper and lower respiratory tract and include but not limited to: reverse transcriptase polymerase chain reaction (rtRT-PCR) and isothermal amplification which includes nicking endonuclease amplification reaction (NEAR), transcription mediated amplification (TMA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HAD), clustered regularly interspaced short palindromic repeats (CRISPR) and strand displacement amplification (SDA).[47] The NAATs has been authorized for different settings, for examples- rtRT-PCR for laboratory setting with trained personnel or some others (isothermal rapid tests) can be performed at POC or even can be self-administered at home or at other non-healthcare locations.[51]

In addition, a cartridge-based nucleic acid amplification test (CBNAAT) GeneXpert, following documented high-level success and wide acceptability in diagnosis of tuberculosis, Cepheid Xpert Xpress SARS-CoV-2 have been authorized for emergency use by the US FDA,[52,53] as well by the WHO.[54] The Xpert Xpress is a 50-minutes RT-PCR-based assay detects the pan-sarbecovirus E gene and the N2 region of the N gene as its SARS-CoV-2-specific target.[52,55,56]

Real time reverse transcriptase polymerase chain reaction (rtRT-PCR):

The rtRT-PCR assay is the gold standard for diagnosis of COVID-19 and is one of the best and accurate laboratory methods for detecting, tracking and studying the SARS-CoV-2 from respiratory specimens, including saliva. This method amplifies a small segment of viral RNA genome, which is converted to cDNA first and then is amplified subsequently. The DNA amplification is monitored in real time using a fluorescent dye or a combination of a quencher molecule and a sequence-specific DNA probe labelled with a fluorescent molecule.[57,58] The most important aspect of this test is that the amplification and analysis are carried out in a closed system, minimising the chances of false positive reactions.[36] A variety of RNA gene targets are used by the manufacturers for one or more of the Helicase (*Hel*), envelope (env), nucleocapsid (*N*), spike (*S*), transmembrane (*M*), RNA-dependent RNA polymerase (*RdRp*) and open reading frame (*ORF1a* and *ORF1b*) genes.[58] In this assay, the viral RNA is measured by cycle threshold (ct)- the number of cycles required for the fluorescent signal to become detectable. A 'ct' value of less than 40 is reported as PCR-positive.[36] Meantime, the WHO published the 'Technical Specifications for Selection of Essential In Vitro Diagnostics for SARS-CoV-2 which includes the series of specifications for SARS-CoV-2 Nucleic Acid Test including intended use (detection target, test purpose, specimen type, testing population etc), performance characteristics (clinical sensitivity->95%, specificity->99%, limit of detection etc), technical and operational characteristics (principle of the assay, specimen stability, turnaround time-4-5 hours, test limitations, etc).[59]

Investigators developed and evaluated a novel, one step nested quantitative real-time PCR (OSN qRT-PCR) for highly sensitive detection of SARS-CoV-2 targeting *ORF1ab* and *N* genes.[60] The sensitivity of OSN qRT-PCR assay was 1 copy/reaction being 10-times higher than that of the commercial qRT-PCR kit (requiring 10 copies/ reaction) and some qRT-PCR negative specimens were detected by OSN qRT-PCR showing higher specificity. Other investigators analysed and validated the OSN qRT-PCR finding it superior showing great potential for detection of SARS-CoV-2, especially in patients with low viral load.[61]

Loop-mediated isothermal amplification (LAMP)

This is a novel method of nucleic acid amplification that can amplify few copies of DNA to 10^9 in less than one hour under isothermal conditions and with higher specificities.[62] Scientists reported in 2020 that a reverse transcription loop-mediated isothermal amplification (RT-LAMP) have been developed for specific detection of SARS-CoV-2 designing the primer set to target the nucleocapsid gene of the virus RNA with detection limit of 10^2 copies of RNA/reaction, which is close to that of qRT-PCR.[63] This test can specifically detect viral RNA of SARS-CoV-2 with no cross-reactivity with currently circulating other coronaviruses, MERS-CoV and other respiratory viruses including influenza viruses. This assay exhibited a rapid detection span of 30-minutes combined with colorimetric visualization and thus the isothermal amplification conjugated with a single tube colorimetric detection may contribute to a simple-to-perform, time-efficient, less expensive yielding high sensitivity and specificity for public health laboratories with limited capacities.

Another group of scientists also developed the RT-LAMP assay for SARS-CoV-2 using a mismatch-tolerant amplification technique and similarly, based on predominantly detection of the N gene.[64] For this purpose, they aligned the SARS-CoV-2 genomic sequence with those of the six other human coronaviruses and several sets of SARS-CoV-2-specific LAMP primers, targeting *N*, *S*, and RdRp genes, were developed. Comparing with a qRT-PCR assay, they found that the SARS-CoV-2-RT-LAMP assay has a high sensitivity and specificity with robust reproducibility and the results can be monitored using a real-time PCR machine or visualized by colorimetric change from red to yellow. The completed reaction time was within 30-minutes for a real-time fluorescence monitoring and 40-minutes for visual detection.

GeneXpert Diagnostics

In line of continuous demand of rapid, easy-to-use at POC, the GeneXpert concept of diagnosis, having previous excellent experiences with tuberculosis, have been considering by a few manufacturers.[52] The first of such test device with a rapid, real-time RT-PCR test for qualitative detection of nucleic acid from SARS-CoV-2 in upper respiratory specimens (nasopharyngeal, oropharyngeal, nasal or mid-turbinate swab or nasal swab/aspirate) was approved for emergency use (Emergency Use Authorization- EUA) by the US Food and Drug Authority on 21 March, 2020 in the name of 'Cepheid Xpert® Xpress SARS-CoV-2' test as well as by the WHO.[53,54]

The Cepheid Xpert® Xpress was then using throughout the globe encouraging many multi-center studies. In one of the multicenter studies in Wuhan, China, investigators reported 96.1% positive percent agreement (sensitivity) and 96.2% negative percent agreement (specificity) with Chinese National Medical Products Administration (NMPA)-approved RT-PCR.[65] Another group of researchers in USA also evaluated the Xpert® Xpress in their multiple centers and found positive agreement of 99.5% and negative agreement of 95.8% with standard-of-care NAATs with a short-time results in approximately 45 minutes.[66] The investigators recommended this technology for the acute-care hospitals in high-prevalent areas, where rapid triage decisions are required for better management of COVID-19 patients. Others also found almost similar results (among them, the UK group found a better agreement) and made similar recommendations.[67,68]

(ii) Serological tests:

Antigen detecting rapid tests

Upon the widespread expansion of COVID-19 infection, a wider proportion of infected community members and a rapidly increasing threat of infecting family inhabitants, there was a strong urge of extending the COVID-19 diagnostic test capacity for a cheaper, faster and easier-to-use at point-of-care (POC). Eventually, rapid diagnostic tests were designed by manufacturers throughout the globe and considering SARS-CoV-2 antigens (spike protein or nucleocapsid) detection by coating corresponding immobilized antibodies on the device.[69] Meantime, the WHO developed a set of technical specifications for selection of SARS-CoV-2 antigen-detecting rapid diagnostic tests (Ag-RDTs), including the detection target (nucleocapsid protein), specimen type (upper respiratory, nasopharyngeal or nasal swabs), test population, intended users, clinical sensitivity and specificity (minimum 80% and 97% respectively).[59]

There could be several different types of Ag-RDT kits like chemiluminescence immunoassay (CLIA), fluorescent immunoassay (FIA), lateral flow immunoassay (LFIA) or lateral flow fluorescent immunoassays- the lateral flow assays being commonly known as immunochromatographic tests (ICTs). The Ag-RDT results can be interpreted without any instrument and available within 10-30 minutes.[59,70,71]

As the Ag-RDTs perform best in individuals with high viral load, WHO recommends that the Ag-RDTs are indicated for the following specific populations and settings: (i) for primary case detection in symptomatic individuals suspected to be infected and asymptomatic individuals at high risk of

COVID-19; (ii) for contact tracing; (iii) during outbreak investigations and (iv) to monitor trends of disease incidence in communities as well as to use the Ag-RDTs that meet minimum performance requirements of >89% sensitivity and >97% specificity.[59]

But the Ag-RDTs are not always promising. In a study in University Hospital Tor Vergata, Rome, Italy during May-September 2020 with 50 nasopharyngeal swabs (collected from emergency department or infectious diseases ward) tested by COVID-19 Ag Respi-Strip (Coris Bioconcept, Belgium), sensitivity of the rapid antigen test was found 30.77%.[72] Whereas, investigators in another study in Thailand during March-May 2020, rapid antigen detecting test prerformed by StarndardTM Q COVID-19 Ag kit (SD Biosensor®, Republic of Korea) from respiratory specimens found a very high sensitivity and specificity of 98.33% and 98.73% respectively. They compared the antigen tests with the real-time RT-PCR test (AllplexTM2019 n-CoV assay (Seegene Korea).[73] The Results of these two studies were also evaluated for diagnostic accuracy by the Cochrane database systematic review. They included forty-eight studies reporting fifty-eight evaluations for antigen tests. They found that estimates of sensitivities were varying considerably among the studies and there were differences between symptomatic and asymptomatic patients (72.0% and 58.1% respectively). Average sensitivity was higher in the first week (78.3%) after symptom onset than in the second week (51.0%) of symptoms. The authors of the Cochrane review also found that the sensitivity was higher in those with PCR cycle threshold (ct) values <25, compared to those with ct values >25 (94.5% vs 40.7%).[74] Nevertheless, an ultra-rapid (within 3 minutes) antigen detection test was found very promising (93.3% sensitivity and 100% specificity) for qualitatively detecting nucleocapsid protein of the virus from nasopharyngeal swabs.[71]

There could be also false positive results with the Ag-RDTs, because of the cross-reacting antibodies embedded with other coronaviruses circulating in a community. These unfortunate false positive results are mostly associated with tests that target nucleoprotein (NP) antigens- whereas, tests that target a highly conserved subunit (S1) of the spike protein of the virus are less likely to yield false-positive reaction. However, mutations in the spike protein occurs frequently among variants of the virus leading to invalidate these test potentialities. Therefore, laboratories and the COVID-19 management team should be careful about the possible false-negative and false-positive results with the Ag-RDTs.[75]

Ag-RDTs for COVID-19 are mostly positive when viral loads are the highest and patients are most infectious- typically 1-3 days prior to and during the first 5-7 days after onset of symptoms.[76]

Antibody detecting assays

Serological tests to detect antibodies (IgA, IgM and IgG) to SARS-CoV-2 have been using in people with active infection and in convalescent cases. Because seroconversion occurs with a median range of 18-21 days after exposure to the virus, the antibody-detecting assays are not suitable for diagnosis of the early stage COVID-19 infections.[77] But the antibody-detecting tests are found very promising especially in low resource countries.[77,78] In a Cochrane database systematic review on antibody tests for identification of current and past infection with SARS-CoV-2 found substantial heterogenicity in sensitivities of IgA, IgM and IgG antibodies- which showed low sensitivities during the first week of symptoms onset, rising in the second week and reaching their highest values in the third week.[79] The combination of IgM/IgG showed pooled sensitivities of 30.1% during 1st week, 72.2% during the 2nd week and 91.4% during the 3rd week. During the next weeks (21 to 35 days), sensitivities for IgM/IgG were 96.0%. Similarly, in an evaluation of performances of two rapid IgM-IgG combined antibody tests, comparing with RT-PCR results, showed 100% specificity and varying sensitivities from 35.7% (0-5 days) to 100% (in patients >15 days of symptoms onset).[80]

Two kinds of antibody-detecting tests are currently available: (i) quantitative antibody detecting enzyme linked immunosorbent assay (ELISA), and (ii) point-of-care qualitative lateral flow chromatographic immunoassays (RDTs).

The ELISA kits are usually based on recombinant nucleocapsid (rN) or spike (receptor-binding domain) (rS) proteins, where ELISA plates are coated with monoclonal mouse anti-human IgG/IgM and subsequent steps including addition of sera specimens, incubation, washing, addition of enzyme (horseradish peroxidase, HRP)-conjugated rN/rS proteins, washing and addition of substrate (tetramethylbenzidine, TMB), incubation and finally reading are similarly employed as for other sandwich ELISAs.[48,81]

In the study by Pan et al, the investigators found similar increasing sensitivities with the days of symptoms onset (11.1%, 92.9% and 96.8% among blood specimens collected during the 1st week, 2nd week and after 2nd week respectively) with colloidal gold-based immuno chromatographic strips targeting SARS-CoV-2 IgM or IgG or both, considering RT-PCR as gold standard with nasopharyngeal swabs from the patients.[78] The rates of IgG detection were higher at all three stages of infection and combined IgM-IgG showed the highest positivity during the intermediate stage (2nd week of symptoms onset).

However, as mentioned earlier, the US Food and Drug Authority (FDA) recommended that the currently authorized SARS-CoV-2 antibody tests should not be used to evaluate a person's level of immunity or protection from COVID-19 at any time, especially after COVID-19 vaccination.[47]

(iii) Microscopy:

The first electron micrograph of a virus (Poxvirus) was published in 1938 and since then, the electron microscope (EM) was one of the first methods to diagnose viral diseases

during the 1940s, and this has been a reliable tool for classification of viruses following their ultrastructure.[82,83] The EM can be applied to many biological specimens and can also hasten routine cell culture diagnosis by observing the growing viruses.[84]

The EM was later associated to virus isolation by cell culture[85,86] and serological methods.[2,87,88] However, after development of the molecular methods of diagnosis, namely real-time quantitative PCR methods or direct nucleic acid extraction associated to next generation sequencing from clinical specimens replaced almost all microscopic tests.

Microscopical identification of SARS-CoV-2 requires either Transmission Electron Microscope (TEM), Scanning Electron Microscope (SEM).[89] However, SEM was proved to be very rapid and efficient tool compared to classical TEM providing a detailed and complete infectious cycle.[90,91] Although, Indian scientists identified the 70-80 nm round virus particles with surface structures on the envelope as morpho-diagnostic features of coronavirus-like particles in the real-time RT PCR-confirmed clinical specimens by TEM, they recommended that imaging thin sections of infected cells by conventional and cryo-ultramicrotomy methods could provide more detailed information that they could not resolve of some interesting features in their images.[92] However, some other scientists in USA earlier used the cryo-electron Microscope to identify the spike glycoprotein trimers of the virus to facilitate medical countermeasure development.[93] The scientists could identify the predominant state of the spike glycoprotein molecule having one of the three receptor-binding domains and they also provided the biophysical and structural evidence that 2019-nCoV (later renamed SARS-CoV-2) spike protein binds angiotensin-converting enzyme (ACE)-2 with higher affinity than SARS-CoV does.

Figure 3 Scanning Transmission Electron Microscope (STEM). Available online at: https://uwaterloo.ca/metrology/tem-stem

In spite of many advantages provided by the electron microscopy, the test procedure has some default limitations for routine usage: (a) requires high costly set up, (b) extensive experience of analysis and interpretation required to rightly identify the virus particles from other cytoplasmic structures in an infected cell.[94,95]

(iv) Culture:

As viruses are obligate intracellular parasites, their propagation requires living cells and viral culture has long been considered as 'gold standard' for diagnosis of viral diseases because it secures an isolate for further analysis.[96]

The value of viral isolation is exemplified by its most significant role in providing epidemiological data, in the diagnosis of new or unknown infections and yielding infectious virions for further study.[20, 97,98] Likewise, the emergence of COVID-19 disease by SARS-CoV-2 was rapidly identified by isolation of the virus by co-culture into VERO cells (kidney epithelial cells of African green monkey) as well as into human airway epithelial cells.[6,98] These isolations rapidly encouraged the testing for antiviral agents' susceptibility and repurposing of newer agents.[98] Further cell lines were also explored and found 6 Simian and one more human cell line (Caco-2) to support growth of the SARS-CoV-2. The cytopathic effects (CPE) were found variable- the lysis of cell monolayer observed within 48-72 hours in the Simian cell lines and no CPE was found in Caco-2 in spite of intense multiplication.[98]

Scientists recently developed a biosafety level-2 cell culture system for production of transcription and replication competent SARS-CoV-2 virus-like particle (trVLP) using the Caco-2 cells. They developed a 96-well format high throughput screening for antivirals discovery and identified some potent antivirals (salinomycin, tubeimoside I, monensin sodium, lycorine chloride and nigericin sodium) against SARS-CoV-2.[99]

Detection of Variants

Several variants of the circulating SARS-CoV-2 are now of great concern (Variants of Concern-VOCs) and monitoring of theses variants are the essential events of management of the pandemic.[30,31,100] Although nucleic acid amplification tests (NAATs), based on reverse transcriptase (rtRT-PCR), are generally considered as a gold standard for detection of SARS-CoV-2 and some of these tests can use one or multiple target genes for amplification, and some of the SARS-CoV-2 VOCs (e.g., Alpha [B.1.1.7] and Omicron [B.1.1.529]) generate a negative (S-gene target failure [SGTF] or significantly weaker positive S-gene result in the RT-PCR assays, some assays that include an S-gene target may fail

detection of the VOCs.[101-106]

Conclusion

Rapid and accurate identification of SARS-CoV-2 is the mainstay of COVID-19 patient management. Sooner the appropriate diagnosis, earlier the healthcare professionals would be able to manage cases of COVID-19 to contain the infection along with its life-threatening complications. Newer methods are currently introducing into every nation increasing capacities of the healthcare workers. It is highly expected that very soon, the pandemic will come to an end with the coordinated management efforts all over the globe.

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