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Review Article

Current and Future Diagnostic Tests for COVID-19 - Challenges and Recommendations

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Abstract

The diagnostic tests for COVID-19 are ranged from molecular tests (detect the RNA virus) to serological tests (detect the past infection). Most of the molecular tests are being developed are based on the real- time RT-PCR assay. For conducting PCR assay the number of molecular targets has been identified within the RNA of Corona Virus; such as helicase (Hel), nucleocapsid (N), transmembrane (M), envelop (E) and envelop glycoproteins spike (S). Serological tests are useful in testing past infection in already recovered patients and the convalescent sera of patients with negative PCR findings. These tests are mostly based on the principles of immunochromatography, chemiluminescence or ELISA to detect IgG or IgG and IgM together in serum samples. Serodiagnosis is useful in testing convalescent sera of patients with negative PCR findings. However, the cross reactivity with other antibodies is a major challenge to serological tests. From the second week IgM titre increases and then gradually declines 3rd week onwards, but IgG level remain stable around week 4.

LAMP, CRISPR and multiplex isothermal amplification followed by the microarray detection methods are being developed around the world, to increase the sensitivity and accuracy for virus detection. LAMP Assay could be a potential alternative to RT-PCR in coming future as a point-of -care device assay. DNA amplifies rapidly in isothermal conditions and this tech doesn't need any specialized equipment. CRISPR-Cas 13 method is also administered, based on specific high-sensitivity enzymatic reporter, unlocks a SHERLOCK protocol for more accuracy and rapidity. NGS is an emerging technology, is used to construct NGS library by amplifying the full length of genes, could be used in epidemiological surveillance and phylogenetic analysis.

These emerging technologies can be implemented to provide better, rapid and accurate diagnosis. These could be used for epidemiological purpose in a wider community, without a need for sophisticated equipment and specific training.

RT- PCR assays help us to tackle this unprecedented outbreak of COVID-19 which has already impacted people's lives and economy. The rapid tests kits are in high demand for providing services in emergency situations and on the bed side of the patients. These tools are best possible tools providing assistance in saving people's lives right on time.

Keywords: *COVID-19; SARSCoV-2; RT-PCR; IgG; IgM; ELISA; LAMP*

Abbreviations

2019-nCoV: 2019-novel Coronavirus; Abs: Antibodies; ARDS: Acute Respiratory Distress Syndrome; BAL: Bronchoalveolar Lavage; BIP: Backward Inner Primer; BOP: Backwards Outer Primer; Bst Polymerase: Bacillus Stearothermophilus Polymerase Enzyme; COVID-19: Coronavirus Infectious Disease-19; CRISPR Cas13: Clustered Regularly Interspaced Short Palindromic Repeats; Cas 13: It is Protein having RNAase Activity to Cleave the RNA; CDC: Centre for Disease Control; Ct: Cycle Threshold; cDNA: complementary DNA; CLIAs: Chemiluminescent Immune Assays; COVIDseq Protocol: Multiplex- PCR; Barcoding and Sequencing (NGS); CLIA: Clinical Laboratory Improvement Amendments; CT scan: computed tomography; DNA: Deoxyribonucleic Acid; E: Envelop Glycoprotein; EUA: Emergency Use Authorization; ELISA: Enzyme Linked Immunosorbent Assay; FDA: Food and Drug Administration; FIP: Forward Inner Primer; FOP: Forward Outer Primer (also called as F3 primer); FRET: Fluorescence Resonance Energy Transfer; gRNA: Guide RNA; Hel: Helicase; Huh-7 Cell: Human Liver Cell Line; LAMP: Loop Mediated Isothermal Amplification; M: Transmembrane; MNPs-magnetic nanoparticles; N: Nucleocapsid; NMT: Nasal Mid-Turbinate; N1/N2/N3: Nucleocapsid Protein Targets; NAAT: nucleic acid amplification technique; Nabs:

Neutralizing Antibodies; NovaSeq 6000: S4 sequencing flow cell; NGS: Next-Generation Sequencing; ORF1a/b: Open Reading Frame 1a/b; ORF1b-nsp14(5'-UTR): Target Sequence for Primers and Probes; OP: Oropharyngeal; qPCR: Quantitative Polymerase Chain Reaction; QIAamp: viral RNA Mini Kit; rRT-PCR: Real-Time Reverse Transcriptase Polymerase Chain Reaction; RdRp: RNA-dependent RNA-polymerase; RdRp/Hel: RNA-dependent RNA-polymerase/ Helicase; RP: RNAase P gene; RT-LAMP: Reverse Transcriptase -Loop Mediated Isothermal Amplification Assay; RT-iiPCR: Reverse Transcriptase Insulated Isothermal Polymerase Chain Reaction; RT-RPA: Reverse Transcriptase-Recombinase Polymerase Amplification; RT-PCR: Reverse Transcriptase Polymerase Chain Reaction; S: spike protein; SHERLOCK: Specific High Sensitivity Enzymatic Reporter UnLocking; SARSCoV-2- Severe Acute Respiratory Syndrome Coronvirus-2; TAT: Turn Around Time; Vero E6: African Green Monkey kidney cells; Vero CCL-81 cells: C- Aethiops Kidney Cells; WHO: World Health Organisation

Introduction

COVID-19 pandemic is an ongoing pandemic, caused by SARSCoV-2. This outbreak was first identified in Dec, 2019 in Wuhan, China. It has affected more than 188 countries in the world. As of 17 August 2020, more than 21.7million people are affected with a death toll of more than 771,378 and more than 14.4 million people have recovered so far. This virus is primarily spread through close contacts, often via small droplets produced by coughing, sneezing and talking.

SARSCoV-2 is an enveloped, non-segmented, positive sense RNA virus which is broadly distributed in humans and mammals. Its diameter is about 65-125 nm, containing single stranded RNA, providing the crown like spikes on the outer surface. SARS CoV-2 is a novel β coronavirus emerged after SARSCoV and MERSCoV outbreaks, which led to pulmonary failure.

Clinical manifestations of COVID-19, are mainly fever, cough and others include shortness of breath and myalgia etc. But some patients have serious complications such as acute respiratory distress syndrome (ARDS), as a consequence of hyper inflammation mainly in lungs due to "cytokine storm", could be fatal in later stages.

The collection of appropriate samples is very crucial to perform RT-PCR. Usually nasopharyngeal swab is taken, but if we miss the detection, then lower respiratory specimens like sputum, bronchoalveolar lavage (BAL) intended for the alternate choice. Currently, various diagnostic tests are available monitoring the prognosis, like immunological (serological) and nucleic acid amplifications tests. Point-of-care molecular devices deliver faster results.

This comprehensive review describes the current/future diagnostic tests, are being used to detect SARSCoV-2 in suspected cases amid pandemic. It is imperative to conduct the correct diagnosis of the disease, while selecting the appropriate samples and availability/utility of various diagnostic tools for tracking the virus to take timely action in controlling the disease transmission.

The challenges in diagnostic evaluation, are ranged from understanding the value of sign and symptoms, predicting possible infection, assessing the existing biochemical and imaging tests can identify infection and whether people need critical care. The new tests deliver the faster results with accuracy. Point -of -care tests are being applied either to identify current infection or to rule out infection or patient needs escalated care or the past infection and immunity.

People with COVID-19 pneumonia (from severe to critical disease) require different patient management, hence It is essential to diagnose the target condition accurately. The disease presents two target conditions as:

- SARS-CoV-2 infection (asymptomatic or symptomatic of any severity)
- COVID-19 pneumonia (severe or critical).

The following two groups must be acknowledged as:

- To identify patients, by performing the existing tests correctly, requiring the respiratory support (SARS or ARDS).
- Identification of asymptomatic host with SARS CoV-2 infection.

Laboratory diagnosis of SARS CoV-2 infection

CDC recommends the upper respiratory specimens like nasopharyngeal specimens are to be collected for RT-PCR based testing, if it is not possible the other specimens such as; oropharyngeal (OP), nasal mid-turbinate (NMT) swab, an anterior nares (nasal-swab) specimen and nasopharyngeal aspirate are to be collected as an alternate option. Similarly, for serological assays the blood samples are collected. The virus can also be found in urine and feces.

The trained healthcare professionals collect the samples; while adhering to the infection control and prevention guidelines of WHO/CDC/and other healthcare bodies All the specimens should be placed in a tube containing transport medium and sent to the lab on time The samples must be transported in triple packaging system; sample vials must be labelled accurately and sealed and the outer covering of absorbent material and then placed in a secondary container and the secondary container should be placed in thermocol under cold temp. All the processes; including packaging, labelling and shipping must be done as per the WHO guideline [4,39].

It is imperative to conclude the accurate final results of highly suspected SARS CoV-2 infected individuals; we should consider collecting various types of specimens to improve detection and cutback the false negative results to monitor the changes in disease prognosis [26].

Virus culture

Virus can be cultured on Vero -cells by inoculating nasopharyngeal and oropharyngeal samples. Cytopathic effects are produced on the 3rd day of inoculation and confirmed by RT- PCR. In Wuhan, 2019-nCoV (SARSCoV-2) is isolated on human airways epithelial cells and Vero E6 and Huh-7 cell lines by inoculating the bronchoalveolar-lavage samples. Cell culture is a laborious task; always require the skilled staff, and virus culture must be performed under the biosafety level-3 facilities only as per the regulatory guidelines. In India, virus is first isolated using Vero CCL-81 cells inoculated with oropharyngeal and nasopharyngeal samples, visualized with cytopathic effects for COVID-19, and observed for coronavirus specific morphology 70-90 nm by transmission electron microscopy [32].

Note: The virus culture for virus isolation for diagnostic purpose, in the epidemic area is not recommended (Licensed commercial/or reference laboratories are the exceptions).

Consideration for specimen types and collection timings

An individual with all relevant manifestations not fully explained by other aetiology should be considered as a suspected case. The diagnosis of suspected and asymptomatic individuals is of a paramount importance in the management and control of the outbreak. When testing is not possible the specimens should be shipped to the reference/or testing labs according to WHO instructions. It is noted that the negative results don't rule out the infection and the tested person may still be on the first day where viral load in the specimen remains undetected at this stage. As a result, cautions and follow up testing are recommended for highly suspected person [18].

WHO interim guide is available for general procedures for specimen collection. Different respiratory specimens from upper and lower parts of the tract, would have different detection rate. This pattern of viral shedding is not fully understood. The accuracy of the tests depends upon specimen quality and quantity, time of collection in the course of disease, and also the inherent quality of kit [48].

The throat swabs obtained found to provide false negative results as the virus load goes undetectable levels, but viral RNA could be recovered from sputum. The lower respiratory specimen collection procedure may increase the risk of getting infection to the medical staff. Hence saliva and nasal wash specimens are found to be as good alternatives. The bedridden patients/those undergo mechanical ventilation, the invasive procedures applied to obtain endotracheal aspirates, sputum or bronchial lavage are the possible ways to obtain the samples [48].

Stool and blood specimens were found to contain SARSCoV-2 and viral RNA has successfully been detected from such specimens by RT-PCR, however the diagnostic value hasn't thoroughly studied. But these procedures can be adopted to test sewage samples for epidemiological purpose could give the indication of active epidemic in a community.

Immediate testing procedures are performed in a biosafety lab 2/or 3. Care should be taken while processing specimens to minimize the aerosol generation. The operators should be aware of technical aspects affecting the accuracy of results. Nucleic acid amplification processing kits reagents should be matched with PCR platform. Reagent preparations and storage are kept according to manufacturer's instructions. RNA extraction process is validated by CDC, can be stored at -70oC, if the subsequent step of analysis is to be performed later. There are two methods for RNA amplification test for SARS CoV-2, rRT- PCR and loop-mediated isothermal amplification (LAMP). rRT PCR method is approved by WHO and FDA, whereas isothermal amplification assays have not been authorized yet. For diagnostic purpose, the RT- PCR is the most common tool

due to its accuracy and popularity. Nonetheless, the accuracy of nucleic acid amplifications is ultimately affected by mutations in sequences targeted by the primers [2,4,5].

Most of the SARS CoV-2 tests are currently approved under the emergency use authorization. The clinical correlation between radiographic screening and test positivity would determine the correct infection status.

Real time RT- PCR

Different qualitative Real Time RT-PCR protocols for SARS CoV-2 diagnosis were developed in various countries. Two diagnostics rRT-PCR based assays are accepted for procurement under emergency use listing procedure; Genesig Real-Time PCR Coronavirus, CE IVD (Primer design Ltd, Southampton, UK) and Cobas SARS CoV-2 6800/8800 system, CE IVD (Roche molecular Systems) [29].

Currently, numerous primers are designed to target various RNA sequences based on six genes of SARS CoV-2 for diagnosis purpose:

- ORF1a/b
- ORF1b-nsp14(5'-UTR)
- RdRp (RNA -dependent RNA-polymerase)
- S (spike protein)
- E (Envelop protein)
- N1/N2/N3 (Nucleocapsid)
- RdRp/Hel (RNA-dependent RNA-polymerase/helicase).

In a recent study nucleocapsid N2 and envelop E genes, to be the most sensitive singleplex reactions, with no significant change in the Ct cycle threshold were noted when both the assays are combined. The rRT-PCR diagnostic recommended by CDC panel includes primers for i) two specific regions of nucleocapsid gene and ii) human RNAase P gene (RP) in one step qualitative RT-PCR based detection [5].

False-negative results may be attributed to various factors:

- Variations in the viral load kinetics may be because of inappropriate sample collection. sometimes it gets negative when we find typical CT scan findings.
- Researchers attributed sensitivity around 70%. SARS genes also undergo mutations and procure active genetic recombinants.

Figure 1: Steps of RT-PCR. RNA is extracted from highly suspected specimen. Transcribed it into complementary DNA (cDNA). Primers annealed to DNA sequence and DNA polymerase help copying it. DNA polymerase degrades the bound probe to increase the fluorescence signals. Fluorescence cross the threshold Ct to test positive in corona positive patients.

- RNA virus generally lacks the efficient proof-reading machinery to ensure fidelity and steadfastness of RNA replication.
- Mutations in primer and probe targeted sequences may lead to false-negative results, but can be reduced by targeting two or three sequences within the viral genome.

Earlier radioactive isotopes were used as marker to detect genetic material, but nowadays various fluorescent dyes are being used as marker. The PCR reactions are carried out in a closed system, therefore chances of getting false positives will be minimized. The real time PCR facilitates in analysing the results in real time even though the process is still ongoing. This molecular testing is still recommended as "gold standard" for relevant case diagnosis [12,34].

Haemagglutinin-esterase, Open reading frame ORF 1a and ORF 1b and RNA dependent RNA polymerase (RdRp), are other genes that encode the structural protein utilized for COVID-19 diagnosis. In real-time PCR the viral RNA is measured by cycle threshold

(Ct) means number of cycles are required to cross the threshold to detect fluorescent signals. The value is less than 40 is clinically reported as PCR positive. Most of RT-PCR are 100% specific, but false negative results may also occur due to sampling error or inappropriate timings of sampling [34].

WHO recommended that the E gene assay followed by confirmatory assay using the RdRp gene can be utilized for the first line screening of COVD-19; and CDC US have also asked to use N1 and N2 two nucleocapsid protein targets in molecular assays. A study published from Hong Kong, explained the detection at even lower limit in case of using the RdRp/Hel *in-vitro* assay. However, it showed the higher specificity and sensitivity among three developed real-time RT-PCR assays targeting the RdRp/Hel, S and N genes of SARSCoV-2 [6].

It is advisable to use two/or more targets to avoid the potential genetic drift of SARS CoV-2 and the cross reaction with other endemic coronaviruses. Ideally one conserved and the other would be the target sequence to mitigate the effect of genetic drift as the virus evolves frequently.

Target Sequences for Real Time RT PCR	Positives Cases	Sensitivity	Specificity
RNA-dependent RNA polymerase-RdRp/ Helicase Hel gene [14]	91%	91%	N/A
Non-structural protein 2(nsp2) [15]	100%	100%	N/A
Open reading frame 1 ab (ORF1ab) [14]	79.4%	79%	100
Open reading frame 1ab (ORF1ab), nucleocapsid(N)gene and envelop (E) gene [12]	63.15%	71%	N/A
Open reading Frame 1 ab (ORF1 ab) [12]	40.98%	N/A	N/A
Nucleocapsid protein gene (NP) gene [20]	39.8%	N/A	N/A

Table 1: Target Sequences used in RT-PCR assay worldwide for the diagnosis of COVID-19.
N/A data Not Available.

Most of the studies conducted using two targets in combination for COVID-19 diagnosis. In a study in Germany, they chose E and RdRpIn. Another study in Hong Kong and China, the researchers used nucleocapsid for screening followed by the ORF1b for confirmation had selected two loci in nucleocapsid gene for good performance [6,9].

Various institutes of Indian Council of Medical Research (ICMR) have evaluated the performance of 31 commercial kits and 14 kits were found to be satisfactory among those [17].

The sensitivity of RT-PCR is reported to be lower than the chest CT examination. However, CT doesn't differentiate between viral and COVID-19 pneumonia.

For accurate diagnosis RT-PCR must be accompanied by computed tomography (CT) radio imaging. The patient develops grounded glass appearance on the chest CT scan before detection of viral RNA. Generally, rRT PCR takes 4-6 days to turn positive after lung manifestations become apparent on radiograph [36].

RNA extraction generally classified into [3]

- One step with RT and the second is with PCR reaction in the same tube.
- Two step RT-PCR (initial creation of DNA copies with RT reaction followed by the PCR reaction).

Typically, one step PCR uses one reaction tube, minimizing the risk of contamination (False positive results). On the other hand, two step PCR allows the cDNA sample to be archived for further testing of other genes.

There is still a risk of false negative results, despite having a validated nucleic acid amplification tests (NAATs). Most of the NAATs relate to the pre-analytic setting, such as the timing of the specimen collection (Early and late samples could limit of detection due to late infections with atypical manifestations). Quality of sampling (insufficient material), types of specimen (bronchoalveolar lavage fluid exhibits more sensitivity, followed by induced sputum, nasopharyngeal swab, oropharyngeal swab etc.), and finally sample transport (inappropriate container, exposure to extreme temperatures etc.) can interfere and compromise with authentic outcome of NAATs [7,33].

RNA extraction method for NAAT	Advancements
rRT- PCR (Real Time Reverse Transcription-Polymerase Chain Reaction)	Reference method, high sensitivity and specificity, compatibility with automation and multipanels. Long TAT (turnaround time) without automation.
Nested PCR	Increased sensitivity due to the added pre-amplification step. Longer TAT and lower specificity due to the higher risk of contamination.
RT-LAMP	Shorter TAT. Possible slightly lower sensitivity
RT-iiPCR	Possible slightly lower sensitivity
Gene Xpert	Automation, high sensitivity and specificity, molecular rapid test high costs, limited number of samples per time.
MNPs-based methods	Increased rapidity, compatibility with automation

Table 2: Advancements of molecular diagnostic methods for SARSCoV-2 detection.

NAAT: Nucleic Acid Amplification Technique; rRT-PCR: Real Time Reverse Transcription Polymerase Chain Reaction; RT-LAMP: Reverse Transcription-Loop Mediated Isothermal Amplification; RT-iiPCR: Reverse Transcription Insulated Isothermal Polymerase Chain Reaction; MNPs: Magnetic Nanoparticles.

If virus mutates frequently could undoubtedly decrease the performance of the tests, by changing the sequence of the region in which primers inhibit the PCR reaction because the alteration in the target sequence. PCR inhibition is observed 0.3% of the tested samples [33].

With the purpose of reducing TAT of NAATs, several rapid microarray and sequencing solutions built on multi-RT-PCR panels have been developed These tests would help rapidly in-patient management especially regarding the isolation procedures [26].

Various manufacturers already developed other specific advanced systems, which are investigated as beneficial in their easiness, rapidity and compatibility with automation [42,47].

Loop mediated isothermal amplification assays (LAMP)

Loop mediated isothermal amplification is based on the technology of auto-cycling strand displacement DNA synthesis via special DNA Bst -polymerase (a large fragment from Bacillus stearothermophilus polymerase enzyme). In such tests, the positive reaction is detected visually or by simple turbidity measurement. Incorporation fluorescent dyes allow the real time monitoring of the reaction. The technique was further developed to enable RNA detection by reverse transcription with successful application in detection of numerous RNA viruses including H7N9 influenza, MERS CoV, West Nile virus and Zika virus. However, these tests are awaiting authorization from WHO and FDA, to be performed even under emergency situation.

LAMP is very sensitive, easy and time efficient method. The LAMP reaction proceeds at a constant temperature using strand displacement reaction. Amplification of DNA via cyclic or non-cyclic amplification steps using 4-6 primers, while the reaction proceeds at a constant temperature using a strand displacement reaction.

Four different types of primers in LAMP assay, are used to recognised six distinct regions of the target gene. The four primers are: Forward inner primer (FIP), Forward outer primer (FOP): The FOP is also called as F3 primer, Backward inner primer (BIP), and Backwards outer primer (BOP).

DNA templates and reagents are incubated at constant temp. 60-65 degree unlike PCR. LAMP primers can anneal with complementary sequence of double stranded target DNA and initiate the DNA synthesis with DNA polymerase with strand displacement activity and releasing the displaced stranded DNA. The following amplification mechanism explains from when the FIP anneals to such released single stranded template DNA.

Successful application of real time LAMP in the SARSCoV-2 pandemic could contribute positively to the pandemic management because this test is rapid giving results within one hour and can be performed in the small instrument facilities at general lab of hospitals/at bedsides/or in the field. However, the designing of a primers for LAMP assay are more cumbersome procedure comparatively comparing to PCR assays.

A handful studies have developed, optimized and attempted RT LAMP assay for the diagnosis of SARS CoV-2 using primers for dif-

Figure 2: Schematic representation of LAMP amplification by initiation, cycling and elongation. Fluorophores are attached to FIP primers and quenched by proximity to guanine bases due to fluorescence resonance energy transfer (FRET).

ferent genes. The primers for the nucleocapsid gene were found to be more sensitive in detecting the 100 RNA copies/reaction and this level is high for sensitive diagnosis of suspected cases. The primer sets of another study targeting ORF/1ab and S-gene have achieved a detection as low as 20 copies/reaction. High sensitivity and specificity of RT LAMP were found to be comparable with PCR [27,44].

Next generation assays

Applications of CRISPR-Cas technology in diagnostic microbiology and biomedicine is increasing rapidly for past few years. A new innovative sensitive diagnostic assay is developed; based on CRISPR-Cas system for the detection of infectious microbes and viruses with minimal or no equipment is required to perform the test [8].

RT-Lamp assay based on CRISPR-Cas system, to develop on a strip to detect the RNA of SARSCoV-2 in nasopharyngeal specimens, have discovered recently by American scientists [1].

The assay is performed by dipping a strip into RNA extracted solution from clinical specimen, and after 40 mins results are to be read visually. The CRISPR Cas13a protein guided by specially designed associated RNA (gRNA) to the base pair of the specific sequence of RNA of SARS CoV-2. When Cas 13a-gRNA complex recognizes the targeted sequence, a labelled single stranded DNA reported probe is cleaved by Cas13a to liberate a fluorescent molecule visible to naked eye. Its current sensitivity is lower than the rRT-PCR. Like nucleic acid amplification assay, CRISPR based diagnostics are also expected to generate false results, if any mutations or changes in the target sequences have occurred.

In qPCR, the inadequate access to reagents and equipment has slowed down the disease detection. The CRISPR Based SHERLOCK (Specific High Sensitivity Enzymatic Reporter UnLocking) technique can advance the diagnosis of COVID-19. This test can be carried out starting with RNA purified from patient samples, as is used for qRT-PCR assays, and can be read out using a dipstick in less than an hour, without requiring elaborate instrumentation. The SHERLOCK COVID-19 protocol works in three steps and can be completed in 1-3 hr [45].

- Isothermal amplification of extracted nucleic acid with the help of recombinase polymerase amplification kit (PRA).
- Incubation and detection of pre-amplified viral RNA sequence using Cas 13a.
- Visual readout of results.

Figure 3: Schematic representation of an assay based on SHERLOCK CRISPR Cas13a to detect SARS CoV-2 and the final results are developed with fluorescent or calorimetric reaction. RT-RPA, reverse transcriptase-recombinase polymerase amplification, C-control sample line, T-test sample line.

Immunodiagnostic approaches

Point of care immunodiagnostic tests, generate rapid results within one hour, are less complex than the nucleic acid tests. Se-

roconversion of SARsCoV-2 patients normally occur in 7-11 days after the onset of symptoms. It is impractical to use antibody tests in the early stage of the infection (i.e. in 2-3 days as Abs are not developed at this stage), nevertheless these tests are useful for retrospective evaluation and epidemiological surveillance, contact tracing and research studies, when we address virus neutralization antibodies. Antibody detecting assays are either run by ELISA, Chemiluminescence or rapid -test lateral flow assays authorized by FDA and WHO.

On the other hand, a single lateral flow cassette was also authorized to detect the nucleocapsid antigen from nasopharyngeal and nasal swabs. Some antibody detecting tests also exploited the recombinant nucleocapsid antigen to detect nucleocapsid antibodies in blood. Dozens of other tests have limited independent validation introduced to the market. Due to high nucleotide sequence similarity with SARS CoV, the cross reactivity is expected to happen and the expected range of sensitivities are from 34% to 80%. The lateral flow rapid kits have the capacity to detect IgG, IgM/ or viral antigen; to make them suitable detecting the current and past infection. These tests reduce time, cost and labour of testing in comparison to nucleic acid amplification assay. The lateral flow assays are based on the principle of immunochromatography as shown in Fig4. However, the sampling variations could affect the outcome of assay [10].

Lateral Flow assays detect IgM and IgG antibodies on site; help us to assess the burden of infections and find out the asymptomatic patients. BioMednomics assay shows 88.66% sensitivity [23]. Utilizing this type of test for antigen detection, could miss the case to detect virus due to variability in sampling and low viral load in the infected person. Serological tests were also used earlier in SARS and other corona outbreaks, played an important role [6].

Immunological tests are used to measure the antibodies in human blood or viral proteins in the respiratory specimens. Antibody detection provides the valuable information as whether a person has been exposed to COVID-19 or not. However, these tests are not conducted for the identification of active cases. The serological tests are done to determine the serological diagnosis for IgM and IgG antibodies. The sensitivity and specificity of IgM antibodies are in 77.3% and 100% and for IgG antibodies are 83.3% and 95% respectively, has been shown by ELISA. Hence, the higher level of specificity in the test makes them more reliable.

Figure 4: Lateral flow assay architecture shows Antigen on the sample pad moves to the next line by adding buffer and attached with conjugate labelled antibodies on conjugate pad and after capillary flow on nitrocellulose membrane they attached with specific primary antibodies (Sandwitch Complex), and become visible to eyes.

The advantage of immunological tests in future is to identify the individuals who have recovered from COVID-19 and also help in choosing the individuals for “convalescent plasma” as treatment option for COVID-19 individuals.

Serological tests are essential to carry out the epidemiological survey due to high no of cases, but these immunological tests have great limitations to apply in the early stage as it will take 6-10 days to develop IgM antibodies, low immune response. WHO has also recommended that these point -of- care immunodiagnostics are used in the research setting, but certainly not to make the clinical decision until unless the other evidences are available to support the diagnosis [39].

Rapid antigen kits are generally characterized by suboptimal sensitivity and specificity. Unique and conserved domains of the proteins in SARS CoV-2 could be exploited to develop sensitive testing kits. Sensitivity can be increased by:

- To concentrate the target antigen before the test.
- Use monoclonal antibodies to different epitopes to be detected.

In a recent study, the immunochromatography of SARS CoV-2 antigen tests, targeting N protein was reported to demonstrate 93-100% sensitivity and 100% specificity.

Serological tests

FDA had granted emergency use licenses to many commercially available serological kits. These tests are mostly based on the principles of immunochromatography, chemiluminescence or ELISA to detect IgG or IgG and IgM together in serum samples. Serodiagnosis is useful in testing convalescent sera of patients with negative PCR findings., as the accuracy of molecular assay is influenced by viral shedding dynamics. However, the cross reactivity with other antibodies is a major challenge to serological tests. In fact, the profile of humoral immune response is still unknown. From the second week IgM titre peaks and increase until the 3rd week and then gradually decreases, but IgG level stabilizes around the 4th week.

Infection of SARS CoV-2 results in cell mediated and humoral immune responses. Humoral response is related to the development of IgM, IgA and IgG uniformly in all patients, except immunodeficient individuals. Serological tests increase the detection rate as the disease is progressed. A recent study showed that ELISA is more accurate for IgG and IgM and lateral flow immunoassay highly sensitive to IgG at the 10th day after infection onset [11,25].

Another immunodiagnostic test of interleukin-6 (Elecsys IL-6 from Roche Diagnostics) has also been granted an Emergency Use Authorization. This test is helpful in determining the risk of intubation with mechanical ventilation as it measures the levels of inflammatory response in individuals with SARS CoV-2.

Serological tests provide a limited sensitivity in the early stages is one of the drawbacks, because host doesn't produce enough antibodies. The delayed response could be associated with more severe disease [19,21,35].

Most of the coronavirus are closely related antigenically. The current virus neutralization test, recommended by WHO, exhibit more than 98% specificity to evade the cross reactivity [39].

Sensitivity and specificity of serological tests are affected by the target antigen, the S-protein (produced in the late stage of infection) shows higher specificity especially in S1 protein than N protein The N protein Abs are decreased earlier as compared to S protein, thus impairing the sensitivity of the test if target N protein only. Therefore, two sets of serological tests are. recommended:

- For sero-epidemiological studies, the tests target the S-protein is highly recommended. Its respective titres reflect the protection against re-infection with same strain.
- The high specificity of S protein study is substantiated in SARS CoV-2 as it exhibits a special S-epitope and this translates the lack of efficacy of SARS CoV against COVID-19 Hence, reinforcing to use target epitope of S-protein.

Figure 5: SARS CoV-2 kinetics- Markers expression during infection and laboratory diagnosis.

There are number of tests available in the market at present. For both NAATs and serological assays, the specificity (>98%) and sensitivity (>95%) should be used. There are various platforms can be used for these assays as; lateral flow assays (LFAs) having lower sensitivity than ELISA and chemiluminescent immune assays (CLIAs).

The median time for seroconversion is 10-14 days, but early seroconversion is also documented as 3-5 days post infection. IgM antibodies appear on the same time as IgG, but IgM lasts for shorter time. The assessment of IgM provides the approximate time of infection [21,35].

More developments are going on point -of -care devices to reduce TAT and increasing no. of tests daily for faster results. Immunochromatographic kits are available in the market.

The diagnosis needs to be done on time, providing the accurate results; currently, the laboratories are required to provide epidemi-

ological information as the magnitude of the epidemic and speed rate of infection. Serological tests become helpful both in clinical diagnosis and for epidemiological purpose to reduce the pressure on labs implicated by molecular diagnostics. Rapid serological assays are more affordable tests, if used correctly, they can cover the large tested population and also help making decision on individuals who didn't showed any symptoms should be treated accordingly followed by virus testing. These tests are adjunctive to the reference methods. Some indications for the serological testing are as:

- Low IgG/IgM levels on 15-day post infection can correlate with immunodeficiency. Hence, help defining the prognosis.
- Molecular diagnosis is still negative despite the suspicious clinical presentations (viz. COVID-19 complications like Kawasaki or Guillain -Barre syndrome, vasculitis, thrombo-embolic etc.) and Late onset disease (meningo -encephalitis or gastroenteritis). The serodiagnosis can solve discrepancies between clinical presentation and RT-PCR results.
- Different tests might be used, together with clinical presentation, as external gold standard to build the positives for validation of new Molecular tests. Serological tests solve discrepancies between the molecular tests.
- Serological tests reflect the SARSCoV-2 infection.
- Molecular tests assess the magnitude and speed of infection of ongoing pandemic, but serological tests show sero epidemiological status.
- Serological tests support the therapy purpose to find individuals with high titre with negative molecular virus, tests could help identify individuals for blood donations for plasma therapy.
- NAATs always remain the reference standard for the diagnosis even in the early stage of the disease, but serological tests are helpful for epidemiological purpose.

NP swab sensitivity can provide false negatives, but samples from lower respiratory tract acquire intubation in patients already suffering from severe respiratory insufficiency. The final decision on the negative NP should always be corroborated by the clinical presentation. Repetition with and serology tests are also required to set the authenticity if patient presents the COVID-19 symptoms.

Perineal swabs could indicate as another represented sample of investigation.

Another considerations weather (respiratory and gastrointestinal) microbiota play a role against diffusion of virus, together with immune system diversity, might contribute to the different clinical presentations and affect viral concentrations [16].

The continuously evolving pandemic allows us to gather more knowledge and investigate on special features of virus and building novel diagnostic approaches with increased sensitivity and specificity. High quality criteria should be established to avoid massive false positives and negatives, despite the dire need to implement NAATs and serological tests. Validation of these tests should be centralized the performance of the tests to save the time.

Next generation sequencing

High throughput approaches for the diagnosis and surveillance for determining the genetic epidemiology have necessitated by COVID-19 pandemic. The COVIDseq protocol used, involved multiplex- PCR, barcoding and sequencing of samples for high throughput detection elucidating the genetic epidemiology of SARSCoV-2. Rahul. C. Bhoyer., *et al.* procured 752 clinical samples in duplicates, were analysed on a single S4 sequencing flow cell on NovaSeq 6000. There was high concordance between the duplicates. In-depth analysis showed that six samples were negative in RT-PCR, but COVIDSeq detected them SARS CoV-2 positive. In addition to that this assay detected SARS CoV-2 in 21 samples and 16 out of them were classified as inconclusive, but found positive using pan-sarbeco probe suggested that COVIDSeq could be used as confirmatory test. This sequencing approach has provided information on the evolution and genetic epidemiology of SARS CoV-2 samples [30].

The Illumina COVIDSeq test is a Next-Generation Sequencing (NGS) *in vitro* diagnostic test on the Illumina NovaSeq 6000 Sequencing System intended to detect SARSCoV-2 virus qualitatively, from nasopharyngeal swabs, oropharyngeal swabs, nasopharyngeal and nasal aspirates, bronchoalveolar lavage (BAL) etc. from suspected COVID-19 individuals by the healthcare provider.

This *in-vitro* diagnostic test used, has been authorized by FDA under an Emergency use authorization (EUA) for use by laboratories certified under the Clinical Laboratory Improvement Amendments (CLIA) of 1988, 42 U.S.C. §263a, to perform high complex tests.

SARS CoV-2 strain present in the sample enable tracking the vi-

rus strain. The test has been designed to sequence up 3072 samples simultaneously using high throughput.

The principle of this test is:

- RNA is extracted using QIAamp viral RNA mini kit.
- Complementary DNA (cDNA) is synthesized using reverse transcriptase with random hexamers.
- The virus genome is then amplified using two separate PCR reactions and they are pooled together.
- The pooled amplified fragments undergo tagmentation. The adaptor-tagged amplicons again go another round of PCR. The indexed libraries are pooled.
- The pooled library is quantified using fluorescent dye.
- The pooled libraries are clustered and sequenced on the NovaSeq6000 sequencing system the NovaSeqXp S4 flow cell workflow. Sequencing by synthesis used labelled dNTP bases are incorporated and during each sequencing cycle the dNTP acts as terminator allow the fluorescent dye imaged to identify and then cleaved to allow next nucleotide. AGTC dNTP are the terminators reversible keep the natural competition to minimize the bias. Base intensity is measures and a quality score is assigned to each base call.
- The DRAGEN COVID Seq Test pipeline analyses sequencing results to detect SARSCoV-2 DNA in the sample. This test performs small variant calling and generates a consensus sequence in FASTA format for research use.

Combined genetic and epidemiological studies have been suggested to provide insights into spread of infection, evolutionary pattern and genetic diversity of the virus and help in effective management and preventive measures. Genomic surveillance is observed as an excellent approach to investigate and regulate COVID-19 transmission [31]. In addition, the sequencing-based methods along with detection, could also provide the advantage of understanding the genetic epidemiology of the outbreak.

Routinely available biomarkers

Many healthcare facilities have access to the standard laboratory tests for infection such as C-reactive protein (CRP), pro calcitonin, measures of anticoagulation, and white blood cell count with differential. Evaluation of these tests in low resource setting could be helpful for trialing people with COVID-19.

Figure 6: SARS CoV-2 Virus genome amplification, fragmentation and post fragmentation QC& library preparation and sequencing.

Figure 7: Bioinformatics and computational sequence alignment, variant calling, SARS CoV-2 genome assembly, multiple sequence alignment and phylogenetic analysis.

Alternative tests

Chest X-ray, ultrasound, and computed tomography (CT scan) are widely used imaging tests to identify COVID-19 pneumonia. There availability and usage vary in different settings.

Rationale

It is essential to understand the tests accuracy and other diagnostic features, which may be used in different settings to establish the diagnostic and management pathways. Estimates of accuracy help inform the diagnostic, screening, isolation, and patient management decisions.

The following protocols are recommended to review the diagnosis as:

- Signs and symptoms presentation.
- Routine lab tests to determine if patient has pneumonia.
- Laboratory based molecular tests.
- CT scanning and other diagnostic images.
- Point- of- care rapid tests for diagnosis.
- Antibody tests for identification of current and past infection.

The review on these tests help providing the consistent information with the evidence of COVID-19 and the accuracy of the tests and presenting characteristics could help understanding the pandemic graph in local area, and also assist in applying control strategies.

Reference standards

Multiple reference standards on their own or in combination have emerged as:

- RT-PCR/rRT-PCR
- RT-PCR, Clinical expertise, and imaging like CT scan of thorax
- Repeated RT-PCR several days apart/or from different samples
- Plaque reduction neutralization assay/or ELISA
- Information at subsequent time point
- WHO or case definitions.

Discussion

Detection of Viral RNA by RT-PCR/or rRT-PCR

Sensitivities of test to the individual genes are comparable according to comparison studies except the RdRp-SARS prime probe, which has slightly lower sensitivity likely due to mismatch in the reverse primer [22].

Viral RNA is measured by cycle threshold (Ct) becomes detectable from the day one and peaks for one week of symptom onset. Ct is required to produce fluorescent signal; lower Ct represents higher viral load. Ct value less than 40 generally considered as PCR positive. This positivity starts to decline by 3rd week and then become undetectable. However, Ct value obtained in severely ill hospitalized individuals is lower than the mild cases. In some mild cases, PCR is still positive after 3rd week, reflects that they don't have the viable virus [41].

In some cases, PCR became positive after 6th week, or some reported positive after two consecutive negative tests, again become positive, suggesting either error in tests/or re-activation of the virus. In a study, 9 patients are attempted to isolate the virus in culture in the first week and in the subsequent week virus culture was not successful which correlates to decline of infection. Symptoms based strategy of CDC US indicates that healthcare workers can return to work, by looking at the improvement in respiratory symptoms and at least 10 days passed since the symptoms first appear [5].

PCR positives decline slowly in nasopharyngeal swabs. In one study, PCR positive in stool observed in 57% patients beyond nasopharyngeal swab by a median of 4 to 11 days. However, it is not related to the clinical severity. Persistence of PCR in sputum and stool was found to be similar as assessed by Wolfel., *et al*. 2020 [41,46].

In a study 205 patients with confirmed COVID-19 infection, RT-PCR positivity was highest in bronchoalveolar lavage specimens (93%), followed by sputum (72%), nasal swab (63%), and pharyngeal swab (32%). False negative results mainly occurred due to inappropriate sample collection especially in nasopharyngeal swabs. False positives occurred due to technical error and reagent contamination too [37].

Detection of antibodies to SARSCoV-2

Serological diagnosis is an important tool for patients who have the symptoms from mild to moderate beyond two weeks of illness

onset. Serodiagnosis help understand the extent of COVID-19 in community and to identify individuals who are recovered and become immune to the virus.

The total antibodies level begins to increase from the second week of disease onset by IgG and IgM ELISA. Seroconversion occurs in patients between third and the fourth week of clinical illness. However, IgM begins to decline by week 4. PCR has higher positivity rate at 5.5 day than IgM, after that IgM starts increasing. Majority of antibodies are produced against NC, hence detecting Abs against NC would enhance the sensitivity. However, the receptor binding domain of S(RBD-S) protein is the host attachment protein, so the Abs against S-RBD are specific neutralizing antibodies. Using one or both antigens (S and NC) the IgG and IgM provides more sensitivity in the tests. However, some Abs especially against NC have the cross reactivity with SARSCoV and possibly other coronavirus too [13,43].

Point of care qualitative tests are available in the market indicate the presence of antibodies. But only the Plaque Neutralization assay can detect the neutralizing antibodies. The high titre of Abs detected by ELISA, can be correlated to the neutralizing antibodies. Long term persistence of these antibodies remains unknown. PCR positivity and antibodies development may vary in children, other groups and asymptomatic individuals.

It is recommended that the real time RT-PCR is used to detect RNA viruses to:

- Deliver rapid and accurate output.
- Guide patient care and management, and
- Guide epidemiological strategies.

Further studies are warranted to define the serological diagnosis with possible correlations between serological response and prognosis.

Interpretation for COVID-19 infection and clinical management

The main aim of this summary is to guide clinical microbiologists, as to correctly use the diagnostics and clinicians to interpret the results accurately.

Real Time RT- PCR remains the "reference method" for diagnosis of SARS-CoV-2 infection despite its varying sensitivity according to the time of infection.

Serology also represents as a valid asset to:

- Try to solve possible discrepancies between a highly suggestive clinical and radiological presentation and negative RT-PCR.
- Solve discrepancies between different PCR assays.
- Epidemiological purpose.

Symptoms	rRT-PCR	IgM	IgG (anti -S)	IgG (anti-N)	Interpretation
+/-	+	-/+	-/+	+/-	Acute Infection
+	-	+	+	+	Recent Infection
+	-	+	+	+	Late onset infection
-	-	-	+	+/-	Old Infection
-	-	-	-	-	Absence of Infection

Table 3: Clinical interpretation based on review summary of rRT-PCR and Antibody tests.
rRT-PCR: Real-Time Reverse Transcription Polymerase Chain Reaction; IgG anti-S: IgG Antibodies Anti Spike; IgG ant-N: IgG antibodies anti nucleocapsid; +/- : Often positive; -/+ : possible to be positive.

Challenges in the molecular diagnosis

Molecular and serological tests were widely compared during the SARS-CoV epidemic previously, have shown the increased sensitivity and specificity in the molecular tests. For this reason, the rRT- PCR represents a validated assay for early diagnosis in patients with suspected SARS CoV-2 infection [39]. The main challenges in molecular diagnosis are:

- To detect small amount of viral RNA to cutback the false negatives.
- To differentiate the positive signals with other pathogens for decreasing the false positives.
- To have large capacity in order to quickly and correctly test a large no of patients, while avoiding the false positives and negatives.

Initially, the diagnosis conducted by targeting S-gene have demonstrated with good specificity (as it differentiates between SARS

CoV and SARS CoV-2), but with limited sensitivity. Sensitivity was further improved by integrating other specific genes of the virus, such as RdRp/Helicase (Hel), Nucleocapsid (N) and Envelop (E) genes. The best results obtained with RdRp/Hel genes, and WHO guidelines recommend the use of RdRp, E, N and S genes in different combinations. Corman., *et al.* have described targeting the E gene, followed by the confirmation with RdRp primers in a fully automated molecular diagnostic platform. RNA is extracted through MagNA Pure 96 System (Roche) and the rRT-PCR was carried out on a Quant Studio 7 System (Applied Biosystems). He found that the E-gene is constantly sensitive than RdRp, performed in 1000 tests. In addition to detecting the possible drift due to mutations and avoid escaping the mutant strain it is advisable to target both E and RdRp genes once a week [6,9,39].

Two diagnostics are in common use for interpretation against COVID-19:

- rRT-PCR (real time reverse transcription - polymerase chain reaction)
- IgM and IgG ELISA (Enzyme Linked Immunosorbent assay).

The variability obtained in these tests is mainly influenced by time series. Therefore, it is imperative to have a correct analysis of tests during different time slots. More and more studies are required to implement these strategies.

It is still a challenging assignment as how to achieve this milestone with correct diagnosis of a positive outcome, by using one diagnostic or combination of diagnostics.

Single diagnostic test doesn't seem to provide the critical information, but a combination of no. of essential diagnostics could eventually help concluding the confirmation of the viral disease. Hence, using variety of tests will lead to more accurate diagnosis by increasing the number of tests.

Normally the variation of the outcome of diagnostics are studied in the adults without being immunocompromised or any comorbidity status, so it is considerable to correlate the diagnosis in terms of detection and length of time since the onset of symptoms. Hence, more studies are required to gather invaluable data set to build a correlation:

- Collection and availability of time series data from different studies.
- Age and gender of patients.
- Existing comorbidities or pre-existing conditions e.g. cancer or patient is having any specific treatment.
- Genetic markers.

Such rich data analysis could be used to predict the probability of a more accurate detection of infections through the range of diagnostic tests.

IgA response must be included; Padoan A., *et al.* have shown a peculiar feature of the Kinetics of IgA and IgM antibodies. They remain persistent over 38 days of follow up from onset of COVID-19. The antigen used in CLIA assay for IgM antibodies are S-antigen and N-protein, while in ELISA S1 specific IgA and IgG antibodies can be detected. Humoral response generated against the respiratory viruses is due to IgA. Patients were analysed for these antibodies, out of 50% had demonstrated clear cut IgA anti-SARS CoV-2 along with IgM and IgG antibodies.

Figure 8: SARS CoV-2 kinetics- Markers expression including a strong response of IgA, during infection and laboratory diagnosis.

The spike binding antibodies targeting S1 is highly correlated to the NAbs. These outcomes could lead to design passive antibody therapy and vaccine development. The further longitudinal investigations on virus specific antibody functions and their protective efficacy over time are highly required [24].

Conclusion

The laboratory-based approach for SARSCoV-2 diagnosis is RT-PCR approved by WHO, CDC US and other countries healthcare

authorities. Due to pandemic situations laboratories were overwhelmed and shortage of reagents became a global issue. The introduction of serological tests will undoubtedly facilitate the pandemic management, while cutting time, costs and workloads of national laboratories and healthcare systems.

Upper respiratory samples are the best choice in the initial infection, but the sputum is most sensitive in later stages. For accurate diagnosis, CT- scan should be done along with rRT-PCR and for proper diagnosis management. Biggest pitfall of the nucleic acid assays are the mutations or any other genetic changes in the RNA virus.

Improvements have also made in point-of -care tests; are expected to assist in better management of pandemic. They are simple to perform and equipped to produce faster results.

Important points about diagnostics are:

- To assess the diagnostic accuracy of signs and symptoms and routine laboratory testing.
- To assess the diagnostic accuracy of RT-PCR and serological tests.

The following factors are considered for the accuracy of the data obtained can be investigated further using meta-regression:

- Laboratory methods (test version), current or past infection, days of symptoms, severity of symptoms, reference standards, sample type, study design, settings etc.
- Specific measurement of biomarkers at every stage.

Next Generation sequencing and microarray analysis are not currently included in routine diagnosis, but these are emerging technologies could be a critical research tools for epidemiological surveillance, and to discover new mutations and virus evolution [19].

The decision about patient treatment and isolation vary according to health services, settings, available resources, and stages of epidemic. These strategies will change overtime when specific treatment and vaccines will arrive. Moreover, diagnostic accuracy and knowledge is also needed to be able to inform decisions.

This review provides the comprehensive knowledge of currently known diagnostics to be used in pandemic for COVID-19 diagnosis. Technical guidance on COVID-19 published by WHO with interim report could help improve continuously. Therefore, it is advised to procure more accurate data regarding the disease and diagnostics at every time.

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