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REVIEW ARTICLE

Laboratory diagnosis of COVID-19: current status and challenges

Bijina J. Mathew[1], Ashish Kumar Vyas[1], Prashant Khare[1], Sudheer Gupta[2], Ram Kumar Nema[2], Shashwati Nema[1], Sudipti Gupta[3], Shivendra K. Chaurasiya[4], Debasis Biswas[1,2], Anirudh K. Singh[1*]

[1]Department of Microbiology, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, India
[2]Regional Virology Laboratory, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, India
[3]Center for Clinical and Translational Medicine, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, Ohio, USA
[4]Department of Biological Science and Engineering, Maulana Azad National Institute of Technology, Bhopal, Madhya Pradesh, India

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ABSTRACT

The magnitude and pace of global affliction caused by Coronavirus Disease-19 (COVID-19) is unprecedented in the recent past. From starting in a busy seafood market in the Chinese city of Wuhan, the virus has spread across the globe in less than a year, infecting over 76 million people and causing death of close to 1.7 million individuals worldwide. As no specific antiviral treatment is currently available, the major strategy in containing the pandemic is focused on early diagnosis and prompt isolation of the infected individuals. Several diagnostic modalities have emerged within a relatively short period, which can be broadly classified into molecular and immunological assays. While the former category is centered around real-time PCR, which is currently considered the gold standard of diagnosis, the latter aims to detect viral antigens or antibodies specific to the viral antigens and is yet to be recommended as a stand-alone diagnostic tool. This review aims to provide an update on the different diagnostic modalities that are currently being used in diagnostic laboratories across the world as well as the upcoming methods and challenges associated with each of them. In a rapidly evolving diagnostic landscape with several testing platforms going through various phases of development and/or regulatory clearance, it is prudent that the clinical community familiarizes itself with the nuances of different testing modalities currently being employed for this condition.

Keywords: COVID-19; SARS-CoV-2; Coronavirus; Diagnosis; COVID-19 nucleic acid testing; Enzyme-linked immunosorbent assay

INTRODUCTION

Coronavirus disease 19 (COVID-19), caused by SARS-CoV-2 virus, is a rapidly spreading global outbreak, that has been recognized as a pandemic by the WHO on March 11, 2020. First reported in Wuhan, China on December 31, 2019 (1), this virus has been responsible for over 76 million confirmed cases and close to 1.7 million deaths as on December 28, 2020 (2). Clinical manifestations of SARS-CoV-2 are highly variable, with majority of infected individuals being asymptomatic carriers (3-5). The symptomat- ic patients may suffer from mild non-specific symptoms like fever, cough, fatigue, runny nose, diarrhea and shortness of breath, or progress to severe respiratory failure requiring support of an intensive care unit and mechanical ventilation (6). The severity of disease is associated with age 60 years and above as well as underlying comorbidities such as chronic lung disease, cardiovascular disease, chronic kid-

*Corresponding author: Anirudh K. Singh, Ph.D, Department of Microbiology, All India Institute of Medical Sciences, Bhopal, Madhya Pradesh, India.
Tel: +91-7049903836
Email: anirudh.micro@aiimsbhopal.edu.in

ney disease, underlying malignancies and diabetes (6, 7).

So far, no satisfactory drug to treat COVID-19 has been introduced and the vaccines are still in Phase III trials. One of the keys to control the spread of COVID-19 is the quick identification of infected individuals for their timely isolation to break the chain of transmission. This makes timely and accurate diagnosis our most useful armor in the fight against this pandemic. As clinical features of SARS-CoV-2 infection overlap with those of other respiratory infections, clinical and radiography-based diagnosis of COVID-19 becomes challenging. In the absence of reliable clinical diagnostic markers, laboratory diagnosis becomes central to the identification of COVID-19 patients. Availability of the whole genome sequence in the very early stages of the pandemic had opened up the floodgate for the development of nucleic acid amplification-based assays such as real-time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) and isothermal nucleic acid amplification assays. While, the rRT-PCR assays have become the mainstay of COVID-19 diagnosis, other nucleic acid-based assays are touted as faster and/or cheaper substitute for it. Furthermore, there is push for the point of care diagnostic methods to expedite case detection. Immunodiagnostic methods such as antigen or antibody based lateral flow assays, also called rapid assays are increasingly in demand. Each of these modalities come with their own set of challenges, wherein molecular methods provide higher specificity and sensitivity but are time consuming and complex, rapid assays offer quicker results at compromised test sensitivity. In this review we present current status of COVID-19 laboratory diagnosis and its challenges.

MOLECULAR DIAGNOSTICS

SARS-CoV-2 belongs to the betacorona virus family and has a 29899 base positive strand RNA genome which encodes 10 proteins. A whole genome BLAST suggests that the virus is closely related to two bat-derived SARS-like coronaviruses, bat-SL-CoVZC45 and bat-SL-CoVZXC21 and displays 88% sequence identity with them across the whole genome. The same strains have also been shown to have similarity of greater than 90% for genes such as E, M, 7, N and 14. However, S gene has the lowest sequence identity (~75%) when compared with these bat-derived SARS-like coronaviruses. Furthermore, SARS-CoV and MERS-CoV genomes are more distant to SARS-CoV2 with an identity of 79% and 50%, respectively. Notably, the SARS-CoV-2 has a similar receptor-binding domain structure with SARS-CoV (8). Moreover, the percent identity of SARS-CoV-2 with human coronavirus strain HCoV-OC43 which causes mild respiratory disease is found to be very low (40.2%) (9). As a whole, the nucleotide and amino acid sequence-based identities of SARS-CoV-2 with other close and known coronaviruses demonstrate its highly divergent nature which requires its own dedi- cated set of diagnostic modalities. Accordingly, sev- eral in-house and commercial nucleic acid tests were developed for COVID-19 diagnosis with rRT-PCR assays being the leading method of choice (10).

Real-time reverse transcriptase PCR (rRT-PCR) assays. RT-PCR is a very sensitive nucleic acid-based technique owing to use of fluorescence for the detection of amplified product. All the RT-PCR based assays for COVID-19 use hydrolysis probe chemis- try for detection. The very first rRT-PCR assay for COVID-19 was developed by Corman and co-workers. They designed primers against RdRp gene, E gene and N gene. E and RdRp gene assays gave more promising results with a limit of detection (LoD) of 5.2 and 3.8 copies per reaction at 95% detection probability, respectively (11). This assay worked with close to 100% specificity. No issues of cross reactivity with other related corona viruses or other respirato- ry viruses were found. Later on, this assay was used as a reference for evaluation of emerging molecular assays from around the world. Nao and co-workers from Tokyo, Japan initially designed a nested PCR targeting ORF1a and spike protein gene of SARS-CoV-2. Later, they also designed an rRT-PCR targeting nucleocapsid gene. Both assays showed no cross reactivity and had sufficient sensitivity with LoD of ~5 copies of RNA per reaction (12). Subsequently, several other state funded laboratories across the world such as Center for Disease Control and Prevention (CDC) USA, CDC China, National Institute of Health, Thailand and Institut Pasteur, France also designed in house rRT-PCR assays. All of these tests target conserved regions of one or more than one of the SARS-CoV-2 genes namely E, S, N, RdRp and ORF1ab. While the amplification of E gene and/or S gene indicates the presence of a Sarbeco virus in the

clinical sample, confirmed diagnosis of SARS-CoV-2 is based on amplification of additional targets such as N gene, ORF1ab or RdRp. Accordingly, most of the rRT-PCR tests are either in a multiplex format including more than one SARS-CoV-2 targets or come as a combination of screening and confirmatory tests (11). Several commercial rRT-PCR assays, based on these principles, are being widely used across the globe. As of December 28, 2020, 73 commercial manual RT-PCR assays have been validated and approved for COVID-19 diagnosis (13).

While manual rRT-PCR is the workhorse of the COVID-19 diagnostics, it is expensive, time-consuming and requires skilled laboratory personnel. Cepheid (US) has developed cartridge-based nucleic acid amplification tests (CB-NAAT), Xpert® Express SARS-CoV-2 which can be run on their current GeneXpert system commonly used for tuberculosis (14). This multiplex rRT-PCR system is designed to amplify E gene and N2 gene and amplification of both E and N2 or N2 only is diagnostic of SARS-CoV-2. Its analytical sensitivity, calculated in terms of LoD, was found to be 0.01 PFU/mL. The primers for E gene also detect human and bat SARS Coronaviruses but N gene assay gave 100% specificity for detection of SARS-CoV-2 (14). Similarly, MolBio Diagnostics, India has developed a chip-based screening assay for β corona viruses which can be run on Truenat system, again a platform developed for tuberculosis diagnosis. These automated closed systems do not require skilled manpower, offer short turnaround time and obviate major biosafety concerns. However, the cost of each test can be prohibitive in resource-constrained settings. Nonetheless, ease of use of such laboratory based automated or point of care/near point of care assays is appealing and understandably as of December 28, 2020,18 such assays have been validated and approved for COVID-19 (13).

Isothermal nucleic acid amplification assays. Other attractive alternatives for rRT-PCR based COVID-19 diagnosis are isothermal nucleic acid amplification assays. One of the major advantages of an isothermal assay is that it does not require an expensive thermal cycler and the assay can be performed isothermally, using much cheaper equipment such as water bath or dry bath. Furthermore, method for visualization can be colorimetric making these assays suitable for point of care diagnostics. Based on the enzymes, set of primers and guiding principle of amplification of nucleic acid and detection methods several isothermal methods such as, loop mediated isothermal amplification (LAMP), Recombinase Polymerase Amplification (RPA), Nicking and Extension Amplification Reaction (NEAR) and Nucleic Acid Sequence Based Amplification (NASBA) have been developed over the time and all of these have been or can potentially be adapted for COVID-19 diagnostics (15, 16). For a detailed review on Isothermal Amplification Assays for COVID-19 please refer to Khan et al. 2020 (16). It is noted that reverse transcriptase LAMP (RT-LAMP) has greater sensitivity and is faster than RT-PCR and understandably several groups have developed one-step reverse transcriptase LAMP for low-cost and faster detection of SARS-CoV-2 RNA in clinical samples. Most of these methods target ORF1a or N gene for detection of SARS-CoV-2 (17-19). The major limitations of these assays are that they are more complex to design and are more likely to give nonspecific results. Nonetheless, low cost, faster turnaround time and comparable sensitivity of these assays make them suitable for community surveillance and as of December 28, 2020, six isothermal amplification-based assay has been approved for COVID-19 diagnosis (13). Recently Feng Zang group at Massachusetts Institute of Technology, combined clustered regularly interspaced short palindromic repeats and CRISPR Associated protein (CRISPR Cas) system with isothermal amplification methods to develop diagnostic assays for infectious diseases called SHERLOCK an acronym for Specific High-sensitivity Enzymatic Reporter Unlocking (20). This has prompted researchers to develop several CRISPR-Cas based assays for COVID-19 diagnosis including SHERLOCK, one-pot visual SARS-CoV-2 detection system (opvCRISPR) (21, 22), All-In-One Dual CRISPR Cas12a (AIODCRISPR) (23), Cas13-based, Rugged, equitable, scalable testing (CREST) (24) and Fn-Cas9 based enzymatic readout for nucleotide detection and nucleobase identification (FELUDA) (25, 26). As the positive reaction for these assays can be visualized on a lateral flow paper dip stick, they can be used as point-of-care molecular diagnostic assays (20).

It is important to remember here that within few months of its emergence, at least three lineages of SARS-CoV-2 have appeared with distinct mutation profiles (6, 27, 28). However, this has not impacted the diagnostic efficiencies and specificities of the currently used assays as primers and probes used in these

assays are designed from highly conserved regions of genes in the SARS-CoV-2 genome (Fig. 1).

IMMUNODIAGNOSTICS

Currently available immunodiagnostic techniques mostly rely on the detection of IgG & IgM antibodies to the viral antigens and offer the benefits of technical simplicity and reduced turn-around time. They have been utilized in epidemiological studies and surveillance programs to estimate the population exposure to the virus. However, the sensitivity of these assays in early vs late phases of illness, their ability to differentiate between acute and remote infection and the cross-reactivity between SARS-CoV-2 and other common coronaviruses remain to be elucidated. In view of these, recommendation of these tests for diagnostic purposes is subject to the demonstration of a specificity of ≥99.5% (29). Nonetheless, as of December 28, 2020, 39 immunoassays have been evaluated and approved for COVID-19 diagnosis (13). Serological tests for COVID-19 diagnosis are broadly of three types: Rapid Diagnostics test (RDT), Enzyme linked immunosorbent assay (ELISA) and Chemiluminescent immunoassay (CLIA).

Rapid diagnostics tests (RDTs). RDTs are an attractive option for large scale testing of COVID-19 in field settings, with results being available in 15-30 minutes. Most RDTs are based on immunochromatography and detect antibodies to the structural proteins, spike (S), nucleocapsid (N), membrane (M), and envelope (E) proteins. RDT designed by US based Cellex Incorporated (Cellex qSARS-CoV-2 IgM/IgG rapid test) is the first US FDA approved (under EUA) serology based test which detects both IgG and IgM antibodies raised against nucleocapsid protein of SARS-CoV-2 from blood, serum or plasma of the patient (30). It claims to have a positive percent agreement (PPA) and negative percent agreement (NPA) of 93.75% and 96.40%, respectively. The first RDT based kit to be approved in China, developed by Guangzhou Wondfo Biotech Co., Ltd., offers total antibody detection and has 86.43% sensitivity and 99.57% specificity (31). Another RDT kit developed by Zhuhai Livzon Diagnostic Inc., that detects IgG and IgM against SARS-CoV-2 from the sample has a sensitivity ranging from 11.1% to 96.8% at different times of testing (0-7 days, 8-14 days or 15 days and more after onset of the symptoms). Apart from antibody based RDTs several antigen detection based rapid kit for diagnosis of SARS–CoV-2 infection has been developed and many are in pipeline (13). Majority of these kits target one of the two major antigens of SARS-CoV-2, spike glycoprotein S and nucleocapsid protein N.

Enzyme-linked immunosorbent assay (ELISA). ELISA tests usually take up to 2-5 hours before reporting the results and unlike RDTs need a dedicated laboratory with sophisticated instrument. Bio-Rad Platelia SARS-CoV-2 Total Ab (Pan Ig), Euroimmun SARS-COV-2 ELISA (IgG), Mount Sinai COVID-19 ELISA Antibody Test (IgG) are some of the FDA approved ELISA tests for SARS Cov-2 diagnosis (32). All three of them have close to 100% specificity and around 95% sensitivity. Among these, Mount Sinai COVID-19 test is a two-step ELISA and has sensitivity and specificity of 92.5% and 100%, respectively. Another kit developed by VITROS diagnostics detects the IgG levels in serum or plasma with a sensi-

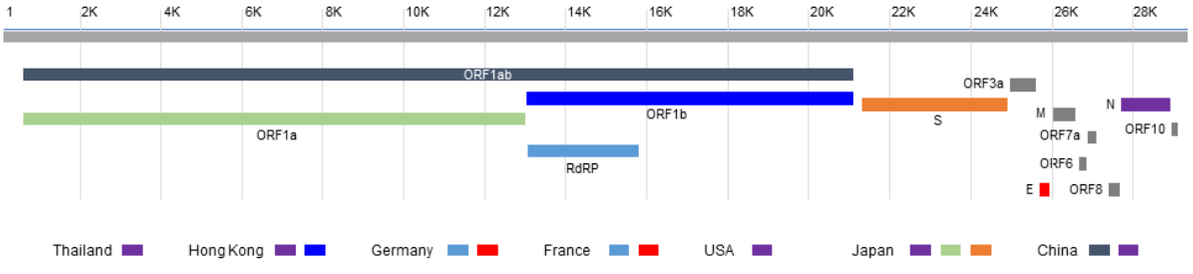

Fig. 1. Genetic organization of SARS-CoV-2 genome and target genes for SARS-CoV-2 molecular diagnosis. Genes/nucleotide fragments depicted in various color bars have been used as targets for developing in-house rRT-PCR diagnostic assays by various national agencies and available at (10). Color bar(s) next to the name of the country represent the gene/fragment used as target for the assays developed there.

tivity of 83% and specificity of 100% (13, 32).

Chemiluminescent immunoassay (CLIA). Similar to ELISA, CLIA also requires sophisticated instruments. However, is faster than ELISA and takes 1-2 hours. DiaSorin LIAISON SARS CoV-2 S1/S2 IgG test, Abbott Architect SARS CoV-2 IgG assay and CLIA kit by Shenzhen YHLO Biotech Co., Ltd are some of the FDA approved tests for use in COVID-19 diagnostics (32). Among these Abbott Architect assay has a sensitivity of 100% and specificity of 99.9% after 17 days from the onset of symptoms (13). Roche has also developed a high throughput CLIA based test (Roche Elecsys Anti-SARS-CoV-2). It detects pan antibodies against SARS-CoV-2 from serum and plasma samples. It has a sensitivity and specificity of 100% and 99.8%, respectively, according to their datasheet. Also, a CLIA based automated analyzer, Caris 200 Automatic Chemiluminescence Analyzer, has been licensed for use in China. Its sensitivity and specificity are calculated to be 94.8% and 99.7%, respectively for detection of total antibody (IgM, IgG and IgA) from serum or plasma sample (33).

CURRENT CHALLENGEE

Threat of COVID-19 is far from over as the various parts of the globe are experiencing the second wave of the spread of SARS-CoV-2. Furthermore, the emergence of a new variant of SARS-CoV-2, supposedly having higher transmissibility has heightened the fear among communities and health care professionals. Ability to provide quick and accurate diagnosis is the key to the management of the current pandemic. One of the major challenges faced by laboratories are to meet the ever-increasing demand of testing. Though considered as the gold standard for COVID-19 diagnosis, rRT-PCR suffers from the limitations of being resource-intensive and time-consuming. Testing of pooled samples has been suggested as an alternative to individualized testing to reduce the cost and turn-around time of the test (34). However, optimal pool size and utility of pooled sample analysis needs to be carefully evaluated for each geographical area as prevalence of the disease can affect accuracy of the diagnosis (35, 36). At the reported point prevalence of 4.77% in a study conducted by us, individual samples with the Ct value of ≥34 were likely to come negative in a pool of five samples leading to false negative results (35). Another limitation of rRT-PCR based diagnosis is use of different SARS-CoV-2 genes as target for identification by different approved IVD assays. Sensitivities and specificities of each available assay vary and no consensus among the scientific and medical community has been reached on the use of a universally accepted COVID-19 diagnostic target. Furthermore, owing to the prolonged and inconsistent duration of PCR-positivity in infected individuals and the poor association between PCR result and viral viability, the current molecular assays cannot be used to monitor the period of transmissibility of a patient and thus decide on the period of isolation (37). Furthermore, inconsistency in collecting respiratory samples also affects the final diagnosis. Less invasive samples such as saliva is being evaluated and the results are encouraging (38). While isothermal assays offer faster diagnosis, many of the limitations associated with rRT-PCR remain unresolved because of the similar nature of the sample and guiding principle of diagnosis at the center of it, i.e. amplification of viral RNA and detection. The currently available immunological assays such as ELISA and CLIA have issue of sensitivity, specificity, turnaround time along with need of sophisticated equipment, therefore, cannot be adopted for large scale diagnosis of COVID-19. Point-of-care tests have the advantages of technical simplicity and brief turnaround time. However, they are yet to demonstrate the desired sensitivity and specificity for reliable diagnosis of COVID-19 and its discrimination from simulating clinical conditions, past infection with common coronaviruses and asymptomatic infection of SARS-CoV-2. Antigen detection tests could fulfill many of these lacunae, but they have not yet been sufficiently evaluated in this condition.

CONCLUSION

While the course of pandemic remains uncertain, the continuing rise in cases warrant that diagnostic laboratory across the globe are ready to meet the demand. Strategies of pooled sample testing by rRT-PCR and advent of quicker and point of care isothermal assays, albeit limitations or specificity and sensitivity offer solution to current need. Immunochromatography based tests are cost-effective, simple and field-adaptable. However, their sub-opti-

mal sensitivity and specificity impair the diagnostic use of the assays currently available (39). Despite these present shortcomings; the concerted efforts of numerous stakeholders and the multi-pronged approaches across the diagnostic landscape, is likely to lead to rapid strides in our journey towards the ideal diagnostic test for COVID-19.

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