Umamaheswari K, et al., J Community Med Public Health Care 2022, 9: 110
DOI: 10.24966/CMPH-1978/1000110

HSOA Journal of Community Medicine and Public Health Care

Research Article

COVID-19: Critical Review on the Clinical Laboratory Investigation Of SARS-Cov-2 Pandemic

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Abstract

The COVID19 pandemic caused by the novel SARS-CoV-2 has led to the unprecedented crisis paving to outrage of the world citizens. The highly contagious SARS-CoV-2 virus claimed to be originated at the Wuhan city, Hubei Province in China, during December 2019 has spread over 216 nations. The COVID-19 pandemic led catastrophe and earlier episodes of similar epidemic SARS outbreaks in China and Middle East interrogate the preparedness in handling such predicament. The major symptomatic manifestations of COVID-19 patients are non-specific and higher incidence of asymptomatic cases often misled diagnosis, unless the CT chest scan findings affirms the disease progress into fatal SARS. Henceforth, robust diagnostic tools are necessitated to confirm suspected cases and to screen large population in less time. Essentially, the clinical laboratories play a pivotal role in COVID-19 testing providing presumptive diagnosis at the earliest and nucleic acid based techniques such as RT-qPCR would provide high fidelity reports. The rapid point-of-care testing is crucial in handling such emergency to enhance large-scale surveillance to aid quarantine of suspicious cases and thereby encasing contagious spread. This review focusses on the challenging prime requisite for accurate laboratory testing of the SARS-CoV-2 infection and the potentials of various techniques in investigating the pandemic.

Keywords: Combined ELISA; COVID-19 testing; EPLEX panel; Immunochromatographic technique; LAMP Assay; RT-qPCR.

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Citation: Umamaheswari K, Balaji S (2022) COVID-19: Critical Review on the Clinical Laboratory Investigation Of SARS-Cov-2 Pandemic. J Community Med Public Health Care 9: 110.

Received: August 29, 2022; **Accepted:** September 12, 2022; **Published:** September 19, 2022

Introduction

The emergence of novel Corona virus 2019 (nCoV) was announced by the Chinese Center for Disease Control and Prevention and later, WHO declared pandemic emergency over the Corona Virus Disease-2019 (COVID-19) outbreak in January 2020. The 2019 nCoV was confirmed of the human-to-human transmission and the cases were evidenced outside China ever since the outbreak, therefore warranting the cause for pandemic [1-4]. As of mid-June 2020 approximately 4,34,796 confirmed deaths and over 7.94 million infected with SARS-CoV-2 virus. The pandemic has resulted in travel restrictions and extensive nationwide lockdowns across countries. The contagious SARS-CoV-2 virus has been involved in acute upper respiratory tract infections that can range from mild to lethal respiratory distress syndrome. Symptomatic cases exhibit atypical symptoms of viral fever, dry cough, dyspnea, sore throat and diarrhea often undistinguished from other viral and bacterial pneumonias. Asymptomatic cases are the major threat by the way of spread to healthcare workers without any prompt signs and symptoms according to WHO [5]. Currently, the situation is worsening as no specific therapies are available for SARS-CoV-2 infection and the prolonged survival of the virus on the inanimate objects is troublesome [6]. In response to the mounting COVID-19 pandemic, molecular testing kits are developed for the detection of SARS-CoV-2 specific proteins and/or nucleic acid in respiratory, serum samples or specific antibodies generated in the host. The continuing spread has led to inadequate testing aids and may miss patients with active infection or falsely categorize patients, further hurdling disease control measures. The delayed and/or failure in probing the outbreak investigation at the start of this contagious viral spread has resulted in extensive morbidity [5,6] Here, we discuss the various means of laboratory testing of COVID-19 and strategies for early and rapid diagnosis.

Members of the CoV Family and Earlier Outbreaks

Corona viruses are the large, pleomorphic, positive sense ssRNA virus with 26-32kbp genome encapsulated in a helical symmetry nucleocapsid (N) protected by an outer envelope (E) with surface characteristics of club-shaped spike (S) and hemagglutinin esterase (HE). The CoVs belong to the family *Coronaviridae,* order *Nidovirales,* and realm *Riboviridae* which includes 4 genera, *Alpha corona viruses* and *Beta corona viruses* infects mammals and *Gamma corona viruses* and *Delta corona viruses* which primarily infect birds [1,7] Ancestrally, the CoV lineages are known to cover a broad host range infecting many mammalian and avian species, very few strains of the CoV family are circulating among humans causes acute respiratory infections. The chronology for human CoVs incidences are as follows: HCoV 229E (1960), HCoV OC43 (1960), SARS-CoV (2003), HCoV NL63 (2004), HKU1 (2005), and MERS-CoV (2012) (37). Historically, only the 2002-2003 major epidemic outbreaks in China due to SARS-CoV originating in bats and in late 2012 the MERS-CoV virus spread from camels to humans in Saudi Arabia are reported of high mortality [5] . Genomic analysis of SARS-CoV-2 revealed 89% and 82% nucleotide homology with bat SARS-like-CoVZXC21 and human SARS-CoV respectively and 40% amino acid identity matched

with the external sub domain of spike's receptor binding domain. Typically the phylogenetic trees of their ORF1a/b, Spike, Envelope, Membrane and Nucleoprotein were also clustered closely with those of the bat, civet, and other hSARS-CoVs [8].

Salient Clinical Features and Host Immune Responses

Understanding the SARS-CoV-2 pathogenesis is imperative in drafting guidelines for screening COVID-19 at the earliest. The typical clinical responses are modulated by combating host immune status from the onset of the virus infestation. The clinical spectrum of COVID-19 varies from paucisymptomatic forms to fatal respiratory to multiorgan failure with systemic manifestations of sepsis, septic shock, and multiorgan dysfunction syndromes [9,10] Fever (88%), dry cough (68%) are the most common signs of COVID-19; other non-specific symptoms includes fatigue, phlegm, anosmia, shortness of breath, myalgia, joint pain, sore throat, headache, chills, vomiting, hemoptysis, and diarrhea that may be confused with other respiratory infections [7]. Radiographic chest examinations (X-ray and CT scan) during the early stage of COVID-19 shows multiple small plaques and interstitial changes in the lung periphery, further deteriorate to bilateral multiple ground-glass opacities, lung consolidation and cardiac injury; the cardinal signs of acute respiratory distress syndrome [4]. Among the COVID-19 cohort studied, the blood and lower respiratory tract specimen tested culture negative for suspected bacteria and fungus in about 76% sepsis patients confirmed SARS-CoV-2 infection only. Viral RNAaemia is chiefly warranted in COVID-19 and therefore along with blood sampling- fecal, urine samples investigations are vital in understanding the pathogenesis and route of transmission [4,11].

The immune response to CoVs follows a typical pattern of IgM and IgG production specifically associated with similar viral respiratory tract infections. The SARS-C0V specific IgM antibodies disappear at 12th week, while the IgG antibody lasts long playing a protective role and specific titer varies during the acute and convalescent phase [12]. Anti SARS-CoV-2 IgM/IgG antibodies are produced over days to weeks after primary infection. The strength of antibody response wave against the COVID-19 depends on several host factors such as age, nutritional status, and immunocompetency [13].The secondary haemophagocytic lymphohistiocytosis hyperinflammatory is often an under recognized syndrome in respiratory infections caused by hCoVs. The salient features include hypercytokinaemia, cytopenias, hyperferritinaemia, and blood clots leading to vascular inflammation, thromboembolism, and hypotensive shock with multiorgan failure [14]. The COVID-19 positive patient blood profile analysis shows high leukocyte count, plasma pro-inflammatory cytokines, serum C-reactive protein, erythrocyte sediment rate and D-dimer. Significantly high levels of pro-inflammatory cytokines, and chemokines included IL1-β, IL1RA, IL7, IL8, IL9, IL10, FGF2, GCSF, GMCSF, IFNγ, IP10, MCP1, MIP1α, MIP1β, PDGFB, TNFα, VEGFA were also observed [7]. These auxiliary findings would play a definite path in leading to progressive declaration on the COVID-19 only when matched with prompt clinical diagnosis.

Laboratory Testing of COVID-19

The current situation demands rapid and high throughput screening as the number of new COVID-19 cases are surging. The WHO protocol for the laboratory investigation of COVID-19 follows the evidence of epidemiological history, clinical manifestations, point of care immunodiagnostic rapid screening for SARS-CoV-2 antigens (E,S,N) and/or antibodies (N/S-IgM/IgG), real time RT-PCR detection of viral RNA, and radiographic Chest imaging (X-ray/CT scan) [15]. Unlikely the atypical clinical symptoms of COVID-19 are misleading, therefore auxiliary examinations such as screening of immune components and nucleic acid detection by real-time reverse transcriptase polymerase chain reaction (RT-qPCR) are recommended followed by convincing identification by CoV blood culture and high throughput sequencing of the whole genome [3,15].

Clinical Specimens

For the biosafety consideration, as per WHO regulations the handling of the specimen should be performed only under the biosafety level 3 conditions until the specimen is rendered non-infectious by disinfecting agents [16]. The accurate diagnosis of COVID-19 involves collection of the correct specimen at onset of the clinical symptoms both for screening and confirmation. The shedding pattern of SARS-CoV studied in 2134 clinical specimens using RT-qPCR revealed the presence of viral RNA in 355 (45%) specimens of nasopharyngeal aspirates followed by 150 fecal specimens (28%) [17]. In a similar examination of 1070 specimens collected from 205 COVID-19 patients, bronchoalveolar lavage fluid specimens showed the highest positive rates (93%), followed by sputum (72%), nasal swabs (63%), fibrobronchoscope brush biopsy (46%), pharyngeal swabs (32%), feces (29%), blood (1%) and none of the 72 urine specimens were tested positive by RT-qPCR targeting the open reading frame *1ab* gene of SARS-CoV-2 [18]. Testing of nasopharyngeal specimens may miss early infection; a deeper specimen may need to be obtained by bronchoscopy which can be overcome by repeated testing as recovery of SARS-CoV-2 in the nasal-pharynx increases with the COVID-19 onset [16]. Exclusively, SARS-CoV-2 RNA are also detected from stool, urine and blood specimens, although less reliable than the respiratory specimens [19]. Specimens collected for laboratory testing of human CoVs are to be maintained at refrigerated temperature for up to 72 hours, or frozen at -70°C or below The oropharyngeal (OP) swabs were used much more frequently than nasopharyngeal (NP) swabs in China during the COVID-19 outbreak; however, the SARS-CoV-2 RNA was detected only in 32% of OP swabs, which was significantly lower than that in NP swabs (63%). CDC recommends collecting the upper respiratory NP swab only and collection of an OP swab is a lower priority, and if collected should be combined in the same tube as the NP swab and transported in viral transport medium [18]. The presence of SARS-CoV-2 was also detected in the initial saliva specimens of eleven COVID-19 patients (91.7%) by RT-qPCR and the median viral load was determined to be 3.3×10^6 copies/ml when cultivated on Vero E6 [20]. For the most sensitive detection of SARS-CoV-2, the collection and testing of both upper and lower respiratory samples such as sputum, bronchoalveolar lavage fluid is recommended. However, the collection of sputum and particularly BAL via bronchoscopy increase biosafety risk to healthcare workers. Advantageously, the upper respiratory specimens are easy to collect, thereby increasing access testing patients with mild symptoms, and can be easily implemented in resource limited settings [10,15].

SARS-CoV-2 cultivation

The SARS-CoV-2 virus has been detected from a variety of upper and lower respiratory sources which includes throat, nasal nasopharyngeal swab, sputum, and bronchial fluid. The viral shedding in the

upper respiratory tract secretions are ideal for SARS-CoV-2 recovery, however the viral load in other specimens varies ideally with the viral pathogenesis[36]. Isolation of HCoVs in cell culture is not routinely performed for diagnostic purposes due to the lack of permissive cell lines, time to results, labor intense and expertise requirements. SARS-CoV and MERS-CoV grows well in primary monkey cells including Vero and LLC-MK2, but cell culture should not be performed for suspected cases in routine diagnostic laboratories specifically for biosafety purposes. The isolation and cultivation of SARS-CoV-2 is permissible only at the authenticated centers for testing. However, virus isolation in cell cultures is critical to obtain isolates for characterization elsewhere to support the development of vaccines and therapeutic agents [10,15].

Immunodiagnostic Methods for COVID-19 Testing

Nano-based rapid card test

Rapid testing kits based on the lateral flow immunoassay suits best on-field large-scale screening for pandemic surveillance, and cost-effective with less detection time. Rapid COVID-19 testing kits are of diagnostic significance in screening during acute phase of infection thereby reducing the need for expensive molecular confirmatory tests. COVID-19 rapid card tests are designed to detect SARS-CoV-2 specific viral antigens E, S, N and/or SARS-CoV-2 specific IgG/IgM antibodies in the respiratory and/or serum specimens [20]. Lateral flow qualitative immunoassay is the key principle for many of the rapid test determining the presence or absence of both anti-SARS-CoV-2-IgM and anti-SARS-CoV-2-IgG clinical specimens. The colloidal gold conjugates bound to the monoclonal probes helps in visualization of the testing [20,21]. The gold nanoprobes bound monoclonal antibody immobilized on the lateral flow strip is highly suited candidate for the rapid and on-site COVID-19 testing.

The comparative sensitivity of three techniques that included IgG/IgM ELISA, colloidal gold-immunochromatographic assay and RT-qPCR in testing COVID-19 reported no significant differences [22]. The combined test kit has a sensitivity of 88.66% and specificity of 90.63%. However, there were still false positive and false-negative results that are dependent on the concentration COVID-19 antibody and the phase of immune response [23]. In a similar comparative investigation on the combinatorial detection of SARS-CoV-2 IgM and IgG using colloidal gold-based immunochromatographic strip along with RT-qPCR produced sensitivity of 11.1%, 92.9% and 96.8% at the early stage (1-7 days after onset), intermediate stage (8-14 days after onset), and late stage (more than 15 days), respectively. The immunochromatographic detection capacity in viral nucleic acid negative suspected cases was 43.6% thereby denoting its high sensitivity potential [20]. Diagnostic indexes evaluated for a similar rapid combined SARS-CoV-2 IgG/IgM showed 91% sensitivity, 95.1% positive predictive value and 82.7% negative predictive value [24].

A total of 535 plasma specimens collected from 173 COVID-19 patients tested for the detection of total IgM/IgG against SARS-CoV-2 and the seroconversion rate for IgM/IgG was found to be 82.7% and 64.7%, respectively. Both IgM and IgG antibodies were detected within 5 days after onset in all 39 COVID-19 patients, thereby recommending serodiagnosis of SARS-CoV-2 infections in the case of inappropriately collected NP swab and disambiguate molecular assays [13]. False-positive results incurred may be due to the sharing of similar epitope with the others members of the CoV family, and other viral etiology of common cold. However, these misinterpretations can be averted with the use of highly specific monoclonal antibody probes, and the other CoVs are not traced to be circulating in the recent times [12,25]. Combination of SARS-CoV-2 RNA and specific antibody detections significantly improved the sensitivity of rapid testing, even in early phase of onset. In exceptional cases, the RT-qPCR confirmed COVID-9 patients exhibiting weak, late or absence of antibody responses. The majority of patients developed antibody response only in the 2ndweek after onset of symptoms; therefore, rapid antibody testing can be validated chiefly in the recovery phase [15,24]. When SARS-CoV-2 was identified, especially when rapid antigen testing and/or molecular assays are neither available nor stable, serology was used as a supplementary diagnostic tool[46]. The sensitivity of testing kits may vary from 34% to 80% and must be validated before use in field testing. In the Indian scenario, as per the ICMR recommendations, the COVID-19 antigen/antibody testing kits authenticated by centralized National Institute of Virology, Pune have been approved for testing and thereby shunning poorly sensitive diagnostic aid [26].

Enzyme Linked Immunosorbent Assay

The point of care quantitative testing of IgM/IgG and ELISA kit for SARS-CoV-2 is promising, yet to be validated for laboratory testing purposes. The combined SARS-CoV-2 nucleocapsid protein specific IgM/IgG ELISA revealed few false-positives with IgG testing by both methods in comparison [21,23]. An ELISA-based IgG antibody detection assay using recombinant HCoV-HKU1 nucleocapsid and spike (S) proteins were developed for testing CoV-HKU1 infections showed steady increasing HCoV-HKU1 seroprevalence from cases of childhood to early adulthood from 0% (<10years of age) to 21.6% (31-40years of age) and the findings were validated with western blot, immunofluorescence analysis and flow cytometry [27,28]. In investigating the SARS-CoV-2 nucleocapsid and spike based ELISA for IgM and IgG testing, 214 COVID-19 confirmed cases revealed 80.4% and 82.2% positive rates for anti-N and anti-S SARS-CoV-2 antibodies and observed an increase in the positive rate for IgM and IgG with an increasing number of days post-disease onset [24]. However, the utility of ELISA in testing of COVID-19 is restricted to sophisticated laboratories as the protocols are heavily laborious and are time consuming.

Chemiluminescence immunoassay

Luminogen based chemiluminescence assay for the detection of anti-SARS-CoV-2 nucleocapsid antibody was developed by Lin *et al* 2020 utilized the recombinant full length nucleocapsid antigens coupled to the tosyl magnetic beads incubated with the test serum and analyzed in an automated chemical immunoluminescence analyzer. A total of 14 false-positive cases (21%) were identified for IgM testing by both ELISA and chemiluminescence assays. Compared to the ELISA kit, a significantly higher detection rate of SARS-CoV was observed in both IgM and IgG by chemiluminescence assay [25].

Molecular Techniques

RT-qPCR, a boon in COVID-19 testing

The genome of SARS-CoV-2 is similar to that of members of other typical hCoVs and contains at least ten open reading frames (ORFs). The first ORF1a/b is translated into two large polyproteins processed to 16 non-structural proteins, which form the viral replicase transcriptase complex in SARS-CoV and MERS-CoV. Other ORFs encode four main structural proteins: Spike (S), Envelope (E), Nucleocapsid

(N) and Membrane proteins (M) [29]. The nucleic acid sequencing based techniques are advantageous over the other diagnostic investigations and are validated as confirmatory testing for COVID-19. The presence of viral RNA does not always reflect acute disease, since RNA traces decrease from 66.7% before day-7 to 45.5% during the days 15th to 39th days of infection [13]. The single negative result for the upper respiratory tract specimen in highly suspected cases does not exclude COVID-19, hence repeated multiple-site sampling testing in combination with assessment of dynamic changes observed in chest imaging are strongly recommended in tracking the progressive disease [30].

COVID-19 testing by RT-qPCR is performed with labeled probes targeting either of RNA-dependent RNA polymerase protein (ORF1 gene), the nucleocapsid protein (N gene), the envelope protein (E gene) and the spike protein (S gene). The Center for Disease Control and Prevention, USA developed a RT-qPCR diagnostic panel for the universal detection of SARS-like Beta corona viruses and specific detection of SARS-CoV-2. Three separate RT-qPCR reactions target the N gene- one primer/probe set detects all the beta corona viruses, while two sets are specific to SARS-CoV-2 are validated and all the three assays should be positive to report presumptive positive for SARS-CoV-2 infection [19]. The Charité testing algorithm for SARS-CoV-2 infection states that two RT-qPCR assays that detects E and RdRp genes needs to be positive in order to progress through the next step. The second step would consist of testing with RT-qPCR targeting SARS-CoV-2 specific RdRp with quality control of *Alpha corona viruses* (CoV-NL63 and 229E) and *Beta corona viruses* (HCoV-OC43, HCoV-HKU1 and MERS-CoV), since other SARS-CoVs are not currently circulating in humans, the cases that are amplified should be considered as true positives for SARS-CoV-2 infections [15]. Use of Chest CT scan in combination with negative RT-qPCR assay in clinical suspicion cases is highly recommended [11]. The possibility of false negatives with a nucleic acid detected by RT-qPCR with high probability is witnessed especially for patients in the early stage of COVID-19 infection [30].

Following the rationale that other human SARS-CoV RNA can be used as a positive control, the RdRp assay with two probes was used as broad range probes reacting with SARS-CoV and novel SARS-CoV-2 and an additional probe that reacts only with novel SARS-CoV-2. By limiting dilution experiments, both the probes whether used individually or in combination, provided the same limit of detection for each target virus. The specific probe RdRP-SARSr-P2 detected only the novel SARS-CoV-2 RNA transcript but not the SARS-CoV RNA. The assays were highly sensitive with E gene and RdRp gene assays detecting 5.2 and 3.8 copies per reaction at 95% detection probability. Similarly the N gene screening assay followed by ORF1b confirmation is important to detect subgenus *Sarbeco virus* [31,32].

Future prospective of COVID-19 testing

The multiplex PCR for the real-time pan-CoV detection targeting four HCoV 229E, HKU1, NL63, and OC43 revealed the diagnostic problem arising through seasonal variation in CoV frequency, and also highlighted on the rate of co-infection [33]. Chu *et al* 2020 reported the use of two monoplex RT-qPCR assays targeting the ORF1b and N gene regions of novel SARS-CoV-2 based on the first publicly available sequence in Genbank along with those for SARS CoV, bat SARS-like CoV and other representative CoVs, the assay was found to have detection limits below 10 copies per reaction [11]. The presence of SARS-CoV-2 in respiratory specimens were detected by RT-qPCR amplification of SARS-CoV-2 ORF1ab, nucleocapsid protein genes fragments for a total of 4880 cases showed 100% positive rate for BAL fluid, 1875/4880 (38.42%) respiratory specimens were positive, 39.80% were positive for SARS-CoV-2 nucleocapsid protein and 40.98% for SARS-CoV-2-ORF1ab [29]. Most of the kits are equipped with three assays with each assay being capable of targeting and detecting a different CoV genes thereby recognize the newer generation arising due to mutation.

Loop-Mediated Isothermal Amplification (LAMP) Assays

LAMP reaction based nucleic acid amplification technique amplifies target DNA with high specificity under isothermal conditions. LAMP assay uses a set of four primers and a DNA polymerase with strand displacement activity to synthesize the target DNA up to 10^9 copies in less than an hour at a constant temperature of 65°C. The final products are stem-loop DNAs with multiple inverted repeats of the target. Isothermal LAMP based method used to amplify a fragment of the ORF1ab gene using 6 primers validated by comparing the sequences with 7 similar CoVs, 2 influenza viruses and 2 normal corona viruses by BLAST were comparable in their sensitivity to the Taqman based qPCR detection method in detecting the newly synthesized RNA equivalent to 10 copies of SARS-CoV-2 [34].

Random-amplification deep-sequencing of the novel CoV by mNGS platform helps in determination its origin, evolutionary history and discrimination among MERS-CoV and SARS-CoV identification. For the diagnostic purposes, the genetic heterogeneity of HCoVs precludes a single pan-HCoV molecular assay while some pan-CoV assays use degenerate primers or multiple primer sets or a single set of non degenerate primers. The respiratory panels consisted multiple sets of oligonucleotide were able to detect the endemic HCoVs (HCoV-NL63, HCoV-HKU1, HCoV-OC43, and HCoV-229E) while SARS-CoV-2 were not detected with these panels. Eleven molecular devices from various manufacturers approved for testing of SARS-CoV-2 revealed variable performances. MGI Tech and Innovita utilized the NGS technique to detect all the species along with SARS-CoV-2 and an isothermal amplification technique coupled with chip detection respectively, while the remaining nine devices utilized real-time technique for amplification and detection [35].

Film array for respiratory virus pathogens

The EPlex Respiratory Pathogen instrument is a comprehensive system requiring no additional accessories and has a testing capacity ranging from 24 to 96 samples tested within 2hrs thus providing flexibility of testing in small spaced laboratories. An added advantage of this system includes the generation of customized reports and potential for bidirectional interfaces with information system of the laboratory[36,37]. The eplex panel was able to detect 4 genotypes on CoV virus (GenMark Diagnostics) performance in a multicentric study with the specimen from US and Canada for the simultaneous detection of 19 viruses that included influenza A virus; influenza A H1 virus; influenza A 2009 H1 virus; influenza A H3 virus; influenza B virus; adenovirus; coronaviruses (HKU1, OC43, NL63, and 229E); human rhinovirus/enterovirus; human meta pneumovirus; para influenza viruses 1,2,3 and 4; and respiratory syncytial virus and two pathogenic bacteria *Mycoplasma pneumoniae* and *Chlamydia pneumonia* revealed that ePlex offered a highly sensitive detection of both the viral and bacterial respiratory pathogens with 100% reproducibility [38-45]. As considered the genotyping information may be utilized for epidemiological studies and may lead to development of treatment strategies [46-49].

Conclusion

The contagious spread of the SARS-CoV-2 demands high throughput processing and the clinical laboratory investigation play a major defensive role to seclude victims away from calamitous COVID-19 pandemic. The accuracy of laboratory reporting are influenced by a number of interrelated factors such as virus shedding, time period of specimen collection, viral load titers, host immune response and age predicts the diagnostic sensitivity of the laboratory practices testing. Adding to the low level of testing centers and inadequacy of trained professional is worrisome inflating the hospital and community spread due to delayed quarantine of the probable missed cases leading to community spread. These challenging variants in the screening and confirmation of SARS-COV-2 infections define the interdisciplinary secular path for coherent laboratory testing to the necessity of standard clinical practices. The future directions leading the mankind in handling such pandemic would be the progress in scientific learning on the advanced diagnostic technologies.

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