EMMERGING VARIANTS OF SARS-CoV-2 & DETECTION BY VARIOUS DIAGNOSTIC APPROACHES

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INTRODUCTION

COVID-19 is an acute respiratory viral illness and extremely contagious infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Affecting the other major organs including respiratory system. Virus responsible for many deaths in worldwide. As the SARS-CoV-2 was declared pandemic, there were multiple new variants responsible including Alpha (3.1.117), Beta (B.1.351), Gamma (P.1), Delta (B.1.617.2), & Omicron (B.1.1.529). For identification of new variants many laboratory tests needed.

Objectives:-

1. This chapter provides useful guidance to laboratories and other stakeholders involved in diagnostics for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).
2. It mainly covers the various aspects of specimen collection, nucleic acid amplification testing (NAAT), antigen (Ag), antibody (Ab) detection and quality assurance.
3. The chapter also throws light on association of COVID-19 and other important infectious diseases such as Hepatitis B, Hepatitis C, HIV, secondary infections, tuberculosis, fungal diseases and nosocomial infections.

BACKGROUND INFORMATION ON SARS-COV-2

The WHO was the first who alert about cluster of pneumonia of an unknown etiology in Wuhan, People's Republic of China on 31 December 2019. The virus was firstly tentatively named in 2019 as novel coronavirus (2019-nCoV). Subsequently the International Committee of Taxonomy of Viruses (ICTV) named it as virus SARS-CoV-2. COVID-19 was named to the illness caused by SARS-CoV-2. SARS-CoV-2 was a devastating effect on the world's population which results in declared to be a global health crisis.

The SARS-CoV-2 was classified between the genus *Betacoronavirus* (subgenus *Sarbecovirus*) of the family *Coronaviridae*. They were enveloped, positive sense, single-stranded ribonucleic acid (RNA) virus with a 30-kb genome. The virus was RNA proofreader mechanism kept the mutation rate relatively low.

The genome encoded the non-structural proteins (some of these were important in forming the replicase transcriptase complex), four structural proteins (spike (S), envelope (E), membrane (M), nucleocapsid (N)) and putative accessory proteins. The virus binded to the angiotensin-converting enzyme 2 (ACE2) receptor for the cell entry.

- **Common human CoVs**: HCoV-OC43 and HCoV-HKU1 (betaCoVs of the A lineage), HCoV-229E, and HCoV-NL63 (alphaCoVs). These viruses can cause common colds and self-limiting upper respiratory tract infections in immunocompetent individuals. However, in immunocompromised and older patients, lower respiratory tract infections can occur due to these viruses.
- **Other human CoVs**: SARS-CoV and MERS-CoV (betaCoVs of the B and C lineage, respectively). These viruses are considered more virulent and capable of causing epidemics with respiratory and extra-respiratory manifestations of variable clinical severity.

There were multiple variants of SARS-CoV-2 had been described, in which a few are considered variants of concern (VOCs), it shows their effect on public health. VOCs are as related to enhance the transmissibility or virulence, decrease in neutralization of antibodies obtained through natural infection or vaccination, the ability to evade detection, or reduction in therapeutics or vaccination effectiveness. Based on the epidemiological update by the WHO, as of by 11 December 2021, five SARS-CoV-2 VOCs had been identified since the beginning of the pandemic:

- **Alpha (B.1.1.7):** first variant of concern described in the United Kingdom (UK) in late December 2020
- **Beta (B.1.351)**: first reported in South Africa in December 2020
- **Gamma(P.1)**: first reported in Brazil in early January 2021

- **Delta (B.1.617.2):** first reported in India in December 2020
- **Omicron (B.1.1.529):** first reported in South Africa in November 2021

All five reported VOCs -Alpha(B.1.1.7); Beta(B.1.351); Gamma (P.1); Delta(B.1.617.2); and Omicron (B.1.1.529) have mutations in the RBD and the NTD, of which N501Y mutation located on the RBD is common to all variants except the Delta variant which resulting in increase in affinity of the spike protein to ACE 2 receptors enhances the viral attachment and its subsequent entry into the host cells. Along with NBD, RBD serves as the dominants the neutralization of target and facilitates antibody production in response to antisera or vaccines. Two recent preprints reported available with single mutation of N501Y singly increase in affinity between RBD and ACE 2 near about ten times more than the ancestral strain (N501-RBD). Interestingly the binding affinity of the Beta (B.1.351) variant and Gamma (P.1) variant with mutations N417/K848/Y501-RBD and ACE 2 was decreased than that of N501Y-RBD and ACE 2.

SARS-CoV-2 Variants of Concern (VOCs)

With the emergence of multiple variants, the CDC and the WHO have independently established a classification system which distinguishes the emerging variants of SARS-CoV-2 into **variants of concern(VOCs)** and **variants of interest(VOIs)**.

- **Alpha (B.1.1.7 lineage)**
    - In late December 2020, a new SARS-CoV-2 variant of concern, **B.1.1.7 lineage**, also referred to as **Alpha variant**, was reported in the UK based on whole-genome sequencing of samples from patients who tested positive for SARS-CoV-2.
    - It was detected by genomic sequencing that the B.1.1.7 variant was identified in a frequently used commercial assay characterized by the absence of the S gene in PCR samples. The B.1.1.7 variant includes 17 mutations in the viral genome. Of these, eight mutations (Δ69-70 deletion, Δ144 deletion, N501Y, A570D, P681H, T716I, S982A, D1118H) are in the spike (S) protein. N501Y shows an increased affinity of the spike protein to ACE 2 receptors, which results in enhancing the viral attachment and subsequent entry into host cells.
- **Beta (B.1.351 lineage)**

- It was reported a new variant of SARS-CoV-2 lineage **B.1.351,** also referred to as **Beta variant** or **GH501Y.V2,** with multiple spike mutations, which resulted in the second wave of COVID-19 infections in Nelson Mandela Bay in South Africa in October 2020.

- The B.1.351 variant includes nine mutations (L18F, D80A, D215G, R246I, K417N, E484K, N501Y, D614G, and A701V) in the spike protein, by which the three mutations (K417N, E484K, and N501Y) are located in the RBD and increasing the binding affinity for the ACE receptors. SARS-CoV-2 501Y.V2(B.1.351 lineage) was reported in the US at the end of January 2021.

- This variant is reported to had an increasing risk of transmission and decreased the neutralization by monoclonal antibody therapy, convalescent sera, and post-vaccination sera.

- **Gamma (P.1 lineage)**
  - The third variant of concern, the **P.1 variant,** also known as the **Gamma variant** or **GR/501Y.V3**, was identified in December 2020 in Brazil and was first detected in the US in January 2021.

  - The B.1.1.28 variant carries the 11 mutations in the spike protein. Three mutations were located in the RBD, similar to the B.1.351 variant Based on the WHO epidemiological update on 30 March 2021, this variant has spread to 45 countries. Significantly, this variant may have reduced neutralization by monoclonal antibody therapies, convalescent sera, and post-vaccination sera.

- **Delta (B.1.617.2 lineage)**
  - The fourth variant of concern, **B.1.617.2,** also called as the **Delta variant,** was initially identified in December 2020 in India and was responsible for the deadly second wave of COVID-19 infections in April 2021 in India. In the United States, this variant was first detected in March 2021 and is currently the most dominant SARS-CoV-2 strain in the US.

  - The Delta variant was firstly considered a variant of interest. However, this variant rapidly spread worldwide, prompting the WHO to classify it as a VOC in May 2021.

  - The B.1.617.2 variant carries ten mutations, in the spike protein.

- **Omicron (B.1.1.529 lineage)**
    - The fifth variant of concern, **B.1.1.529**, also designated as the **Omicron variant** by the WHO, was first identified in South Africa on 23 November 2021 after an uptick in the number of cases of COVID-19.
    - The **Omicron (B.1.1.529)** became the dominant VOC in many countries, and many subvariants were identified.
    - The Omicron VOC is currently the dominant SARS-CoV-2 variant in the US, according to the CDC.

The SARS-CoV-2 is the seventh coronavirus identified that is known to infect humans (HCoV). Four of those viruses, HCoV-229E, HCoV-NL63, HCoV-HKU1 and HCoV-OC43, were endemic, seasonal and tend to cause mild respiratory disease.
The other two viruses are the more virulent zoonotic Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus type 1 (SARS-CoV-1). SARS-CoV-2 is most genetically similar to SARS-CoV-1, and both of those viruses belong to the subgenus *Sarbecovirus* within the genus *Betacoronavirus*.

- According to the CDC, conditions with conclusive evidence demonstrating higher risk include:
    - Asthma
    - Cancer
    - Cerebrovascular disease
    - Chronic kidney disease
    - Bronchiectasis
    - COPD (Chronic obstructive pulmonary disease)
    - Interstitial lung disease
    - Pulmonary embolism
    - Pulmonary hypertension
    - Cirrhosis

- Nonalcoholic fatty liver disease
- Alcoholic liver disease
- Autoimmune hepatitis
- Cystic fibrosis
- Diabetes, type 1 and 2
- Heart conditions (such as heart failure, coronary artery disease, or cardiomyopathies)
- HIV (Human immunodeficiency virus)
- Mental health conditions such as mood disorders and Schizophrenia spectrum disorders
- Obesity (defined as body mass index (BMI) of greater than 30 kg/m^2 or greater than 95th percentile in children)
- Pregnancy and recent pregnancy
- Smoking, current and former
- Solid organ or blood stem cell transplantation
- Tuberculosis
- Use of corticosteroids or other immunosuppressive medications (CDC: Underlying Medical Conditions Associated with Higher Risk)

However, SARS-CoV-1 is now not known to circulate in the human population.

The clinical presentation of SARS-CoV-2 infection can range from asymptomatic infection to severe disease.

Clinical Manifestations of COVID-19

- The median incubation period for SARS-CoV-2 is estimated to be 5.1 days, and most patients can develop symptoms within 11.5 days of infection.
- The clinical spectrum of COVID-19 varies from asymptomatic forms to clinical illness characterized by acute respiratory failure requiring mechanical ventilation, septic shock, and multiple organ failure.
- It is estimated that 17.9% to 33.3% of infected patients will remain asymptomatic.
- Most symptomatic patients present with fever, cough, and shortness of breath. Less common symptoms include sore throat, anosmia, dysgeusia, anorexia, nausea, malaise, myalgias, and diarrhea. According to a report that there were 373,883 confirmed symptomatic COVID-19 cases in the USA, 70% experienced fever, cough, and shortness of breath, 36% reported myalgia, and 34% reported headache.
- In a large meta-analysis evaluated that the clinicopathological characteristics of 8697 patients with COVID-19 in China reported laboratory abnormalities that included

lymphopenia (47.6%), elevated C-reactive protein levels (65.9%), elevated cardiac enzymes (49.4%), and abnormal liver function tests (26.4%). Other laboratory abnormalities included leukopenia (23.5%), elevated D-dimer (20.4%), elevated erythrocyte sedimentation rate (20.4%), leukocytosis (9.9%), elevated procalcitonin (16.7%), and abnormal renal function (10.9%).

- An another meta analysis of 212 published studies with 281,461 individuals from 11 countries/regions reported that severe disease course was noted in about 23% of the patients, with a mortality rate of about 6% in patients infected with COVID-19.
- With an elevated neutrophil-to-lymphocyte ratio (NLR), an elevated derived NLR ratio (d-NLR), and an elevated platelet-to-lymphocyte ratio indicate a cytokine-induced inflammatory storm.

Based on the severity of the presenting illness, it involves clinical symptoms, laboratory and radiographic abnormalities, hemodynamics, and organ function, the National Institutes of Health (NIH) issued guidelines that classify COVID-19 into 5 distinct types.[NIH COVID-19 Treatment Guidelines]

- **Asymptomatic or Presymptomatic Infection**: Individuals with positive SARS-CoV-2 test without any clinical symptoms consistent with COVID-19.
- **Mild illness**: Individuals who have symptoms of COVID-19, such as fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, anosmia, or dysgeusia but without shortness of breath or abnormal chest imaging.
- **Moderate illness**: Individuals with clinical symptoms or radiologic evidence of lower respiratory tract disease and oxygen saturation (SpO_2) ≥94% on room air.
- **Severe illness**: Individuals who have SpO_2 less than 94% on room air, a ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (PaO_2/FiO_2) of less than 300, marked tachypnea with a respiratory frequency of greater than 30 breaths/min, or lung infiltrates that are greater than 50% of total lung volume.
- **Critical illness**: Individuals with acute respiratory failure, septic shock, or multiple organ dysfunction. Patients with severe COVID-19 illness may become critically ill with the development of acute respiratory distress syndrome (ARDS). This tends to occur approximately one week after the onset of symptoms.

ARDS is characterized by a severe new-onset respiratory failure or worsening of an already identified respiratory picture. The diagnosis required bilateral opacities (lung infiltrates >50%), not fully explained by effusions or atelectasis. The Berlin definition classifies ARDS into 3 types based on the degree of hypoxia, with the reference parameter being PaO_2/FiO_2 or P/F ratio:

- **Mild ARDS**: 200 mm Hg $< PaO_2/FiO_2 \leq 300$ mm Hg in patients not receiving mechanical ventilation or in those managed through non invasive ventilation (NIV) by using positive end-expiratory pressure (PEEP) or a continuous positive airway pressure (CPAP) ≥5 cm H2O.
- **Moderate ARDS**: 100 mm Hg $< PaO_2/FiO_2 \leq 200$ mm Hg
- **Severe ARDS**: $PaO_2/FiO_2 \leq 100$ mm Hg

When PaO_2 is unavailable, a ratio of $SpO_2/FiO_2 \leq 315$ suggests ARDS. A multi - center prospective observational study that analyses the 28-day mortality in mechanically ventilated patients with ARDS concluded that COVID-19 patients with ARDS had features same as other ARDS cohorts, and the risk of 28-day mortality increased with ARDS severity.

Extrapulmonary Manifestations of COVID-19

- **Neurologic manifestations**: Besides anosmia and ageusia, other neurological findings include headache, stroke, impairment of consciousness, seizure disorder, and toxic metabolic encephalopathy. Five patients with COVID-19 developed Guillain-Barré syndrome (GBS) based on a case series report from Northern Italy.
- **Cardiac manifestations:** Myocardial injury manifesting as myocardial ischemia/infarction (MI) and myocarditis are well-recognized cardiac manifestations in patients with COVID-19. Other common cardiac manifestations include arrhythmias, cardiomyopathy, and cardiogenic shock. A single-center retrospective study analysis of 187 patients with confirmed COVID-19 reported that 27.8% of patients exhibited myocardial injury indicated by elevated troponin levels. According to a study it had been noted that patients with increased troponin levels had more frequent malignant arrhythmias and a high mechanical ventilation rate compared with patients with normal troponin levels. Pre-existing cardiovascular disease seems to be associated to come with more adverse outcomes and elevated risk of death in patients with COVID-19.
- **Hematologic manifestations:** Lymphopenia is a common type of laboratory finding in a large majority of patients with COVID-19. Other laboratory abnormalities include thrombocytopenia, leukopenia, elevated ESR levels, C-reactive protein (CRP), lactate dehydrogenase (LDH), and leukocytosis. COVID-19 is also linked to a state of coagulopathy as evidenced by the high prevalence of venous and thromboembolic events such as PE, DVT, MI, ischemic strokes, and arterial thromboses that also occurred in patients despite being maintained as prophylactic or even therapeutic systemic anticoagulation. Notably, COVID-19 is associated with marked increased level of D-dimer, fibrinogen levels, prolonged prothrombin time (PT), and partial thromboplastin time(aPTT) patients at risk of developing arterial and venous thrombosis. Clinical trials are needed to define the benefit of therapeutic anticoagulation in patients with COVID-19, especially at what stage of the illness.
- **Renal manifestations:** Patients hospitalized with severe COVID-19 are at risk for developing kidney injury, most commonly manifesting as acute kidney injury (AKI), which is likely to be a multifactorial in the setting of hypervolemia, drug injury, vascular injury, and drug-related injury, and possibly direct cytotoxicity of the virus itself. AKI is the most frequently encountered extrapulmonary manifestation of COVID-19 and is associated with increased mortality risk. A large multicenter of cohort study of hospitalized patients with COVID-19 that involved 5,449 patients admitted with COVID-19 reported that 1993(36.6%) patients developed AKI during their hospitalization, of which 14.3% of patients required renal replacement therapy(RRT). Other clinical and laboratory manifestations include proteinuria, hematuria, electrolyte abnormalities such as hyperkalemia, hyponatremia, and acid-base balance disturbance such as metabolic acidosis.
- **Gastrointestinal manifestations:** Based on a meta-analysis by Elmunzer et al.; that involved 1992 patients across 36 centers,1052 patients (53%) experienced GI symptoms, with the most common reported symptoms being diarrhea (34%), nausea

(27%), vomiting (16%), abdominal pain (11%). Cases of acute mesenteric ischemia and portal vein thrombosis have also been described.

- **Hepatobiliary manifestations:** Elevated level of liver function tests can be frequently be noticed in 14% to 53% of patients with COVID-19 infection. Hepatic dysfunction causes more frequently in patients with severe COVID-19 illness.
- **Endocrinologic manifestations:** Patients with pre-existing endocrinologic disorders such as diabetes mellitus are at increased risk of developing severe illness. Clinical manifestations were like to be abnormal blood glucose levels, euglycemic ketosis, and diabetic ketoacidosis have been noted in patients hospitalized with COVID-19.
- Acral lesions resembles to pseudo chilblains (40.4%) are the most common cutaneous manifestation noted in patients with COVID-19.
- Other cutaneous manifestations include erythematous maculopapular rash (21.3%), vesicular rashes (13%), urticarial rashes (10.9%), vascular rashes (4%) resembling livedo or purpura, and erythema multiforme-like eruptions (3.7%).

The Mortality rates were differ from country to country. Early laboratory diagnosis of a SARS-CoV-2 infection can results in timely clinical management and prevention of its further transmission.

The diagnostic testing mainly depends on detection of the virus itself (viral RNA or antigen) or detecting the human immune response to infection (antibodies or other biomarkers).

While our understanding of SARS-CoV-2 has rapidly expanded with the progression of pandemic, however there are many outstanding questions still pending for their addressal by scientific community across the world.

The World Health Organization declared COVID-19 as a global pandemic on March 11, 2020 (WHO 2020c). The disease primarily spreads via close contact of respiratory droplets generated by infected individuals (Center for Disease Control and Prevention 2020a).

COVID-19 is a type of systemic viral illness based on its involvement in multiple major organ systems.

- Patients at higher age group and comorbid conditions such as obesity, diabetes mellitus, chronic lung disease, cardiovascular disease, chronic kidney disease, chronic liver disease, and neoplastic conditions are at risk of developing severe COVID-19 and it is related to complications. The most common complication of severe COVID-19 illness is associated with progressive or sudden clinical deterioration leading to acute respiratory failure and ARDS or multiorgan failure leading to death.
- Patients with COVID-19 illness were also at higher risk of developing prothrombotic complications such as pulmonary embolisms, myocardial infarctions, ischemic strokes, and arterial thrombosis.

- Cardiovascular system involves results in malignant arrhythmias, cardiomyopathy, and cardiogenic shock.
- GI complications such as bowel ischemia, transaminitis, gastrointestinal bleeding, pancreatitis, Ogilvie syndrome, mesenteric ischemia, and severe ileus are often noted in critically ill patients with COVID-19.
- Acute renal failure is the most common type of extrapulmonary manifestation of COVID-19 and is related to an increased mortality risk.
- In a meta-analytic study of 14 studies evaluating the prevalence of disseminated intravascular coagulation (DIC) in hospitalized patients with COVID-19 reported that DIC was observed in 3% (95%: 1%-5%, P <0.001) of the included patients. Additionally, DIC was noted to be related to severe illness and was a poor prognostic indicator.
- More recent data had emerged regarding prolonged symptoms in patients who had recovered from COVID-19 infection, termed "post-acute COVID-19 syndrome." A large cohort study of 1773 patients performed 6 months after hospitalization with COVID-19 revealed that most exhibited at least one persistent symptom: fatigue, muscle weakness, sleep difficulties, or anxiety. Patients with severe illness also had an increased risk of chronic lung issues.
- In a retrospective cohort study that includes 236,379 patients reported substantial neurological (intracranial hemorrhage, ischemic stroke) and psychiatric morbidity (anxiety disorder, psychotic disorder) 6 months after being diagnosed with COVID-19.
- By a secondary invasive fungal infections such as COVID-19 is related to the pulmonary aspergillosis and rhino-cerebro-orbital mucormycosis had been increasing was b reported as complications in patients recovering from COVID-19. Risk factors for developing secondary fungal infection include comorbid conditions such as uncontrolled diabetes, associated lymphopenia, and excessive use of corticosteroids.

At the global level, testing capacity is not sufficient for COVID-19 is till now available as it should be and therefore prevents the individuals from accessing care. During the initial outbreak period, different countries have followed and implemented various testing strategies, depending on the availability of diagnostics and consumables.

However, strict steps taken by the WHO has made the diagnostic available with the mission to “detect, protect and treat” to break the chain of transmission of SARS-CoV-2 (WHO 2020).

Therefore, early diagnosis and prompt treatment can substantially decreases the number of prospective cases. Hence, laboratory diagnosis of SARS-CoV-2 holds the key in containing and restricting the COVID-19 pandemic.

The people who were in close contact with suspicious exposure had advised to be under a 14-day health observation period that should be started from the last day of contact to infected individuals.

Once of those individuals showed any symptoms includes coughing, sneezing, shortness of breath or

diarrhoea, they should required an immediate medical attention. Immediately the isolation of the suspected individual need to be perform with proper guidelines, and they should be closely monitored for clinical symptoms and diagnosis should be performed in hospital-based laboratories as soon as possible.

Moreover, the surveillance must be perform on those who are in contacted with the suspect and some confirmed cases by observing their symptoms clinically. Before taking any decision about isolation, authorities would make sure that whether the suspected case requires home isolation and with careful clinical evaluation with some safety protocol to assess by healthcare professionals or not.

If the suspected individuals presented with any symptoms during isolation, they would contact to doctors as soon as possible for treatment.

During home isolation, there were suggestion for medication and symptoms should be recorded closely. The suspected, probable and confirmed case definition of COVID-19 by the WHO had presented in Fig. 9.1.

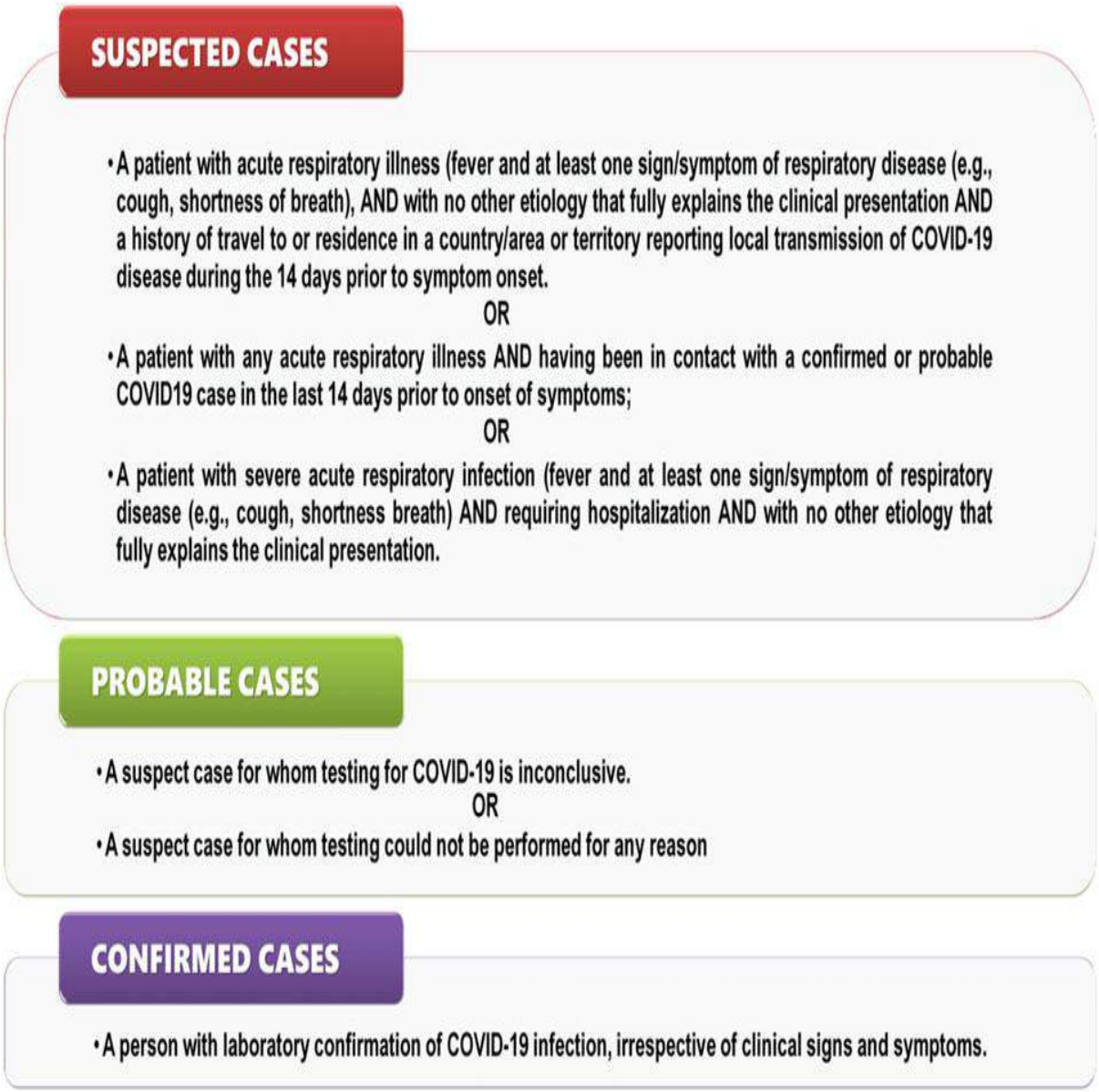


Fig. 9.1 Case definition of COVID-19 by the World Health Organization.

Diagnostic approaches for the detection of SARS-CoV-2 infection

Laboratory virology tests are important for an exact diagnosis and the population level that shows the prevalence rate of COVID-19, shows the number of asymptomatic cases or not specific symptoms that were shown clinically. The results from the tests guided clinicians and healthcare officials were there for management, control, and prevention of COVID-19.

There were the principals which were of two types of tests that were available for COVID-19: the viral tests and the antibody tests. The viral tests that were direct tests as they were designed that detected the virus and as they were reflected in current infection. In contrast, the antibody tests are indirect tests, as they did not detected the virus, but rather than some certain established seroconversion of previous infection, or early seroconversion to current infection.

Direct- The recommended tests for diagnosis of SARS-CoV-2 infection involving detection of viral RNA connected to nucleic acid amplification tests (NAAT), like reverse transcription (RT)-PCR; Certain antigen detection tests should be designed to directly identify the viral particles that were present in biological samples like nasopharyngeal secretions.

- **Indirect-** In contrast to NAAT-based testing they were immediately be in sequence to be known as diagnostic tests were built, the diagnostic technology and methodology underlined the development of some serological tests were likely to be different, with some substantiality to be with the longer timeline to be obtained to be a robust product that would be suitable for routinely deployment

Direct-

- Direct demonstration of the Viruses
  - Transmission electron microscopy imaging of SARS-CoV-2
  - Scanning electron microscopy imaging of SARS-CoV-2
- Rapid Antigen Test (RAT)
- Isolation of virus
- Biosensors
- Aptamer based nano-biosensor
- Paper based detection

Molecular Methods to detect viral genes-

- Reverse Transcriptase-qualitative Polymerase Chain Reaction (RT-qPCR)
  - **Taqman-based RT-qPCR detection**
  - **SYBR green RT-qPCR detection**
- Truenat
- Cartridge Based Nucleic Acid Amplification Test (CBNAAT)
- Nested RT-PCR
- Semi Nested RT- PCR
- Isothermal Amplification Technologies (IAT)

- Reverse Transcription Loop-mediated Isothermal Amplification (RT-LAMP)
- Recombinase Polymerase Amplification (RPA)
- Nicking Enzyme Assisted Reaction (NEAR)
- Biofire FilmArray Respiratory Panel (RP)
- Next-generation sequencing (NGS)
- Nanopore Targeted Sequencing (NTS)
- CRISPR-based SHERLOCK

Indirect-

- **Detection of specific antibodies**
- Enzyme-linked immunosorbent assays (ELISA)
- Immunofluorescence assays (IFA)
- Lateral flow assays (LFA)
- Chemiluminescence enzyme immunoassays (CLIA)
- Magnetic chemiluminescence enzyme immunoassay (MCLIA)

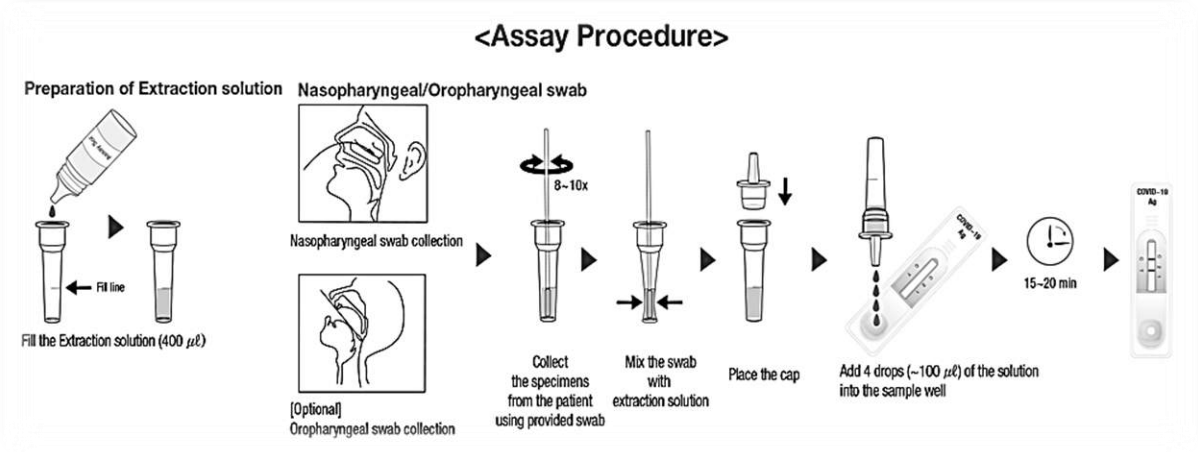
Other Laboratory Diagnostic tests

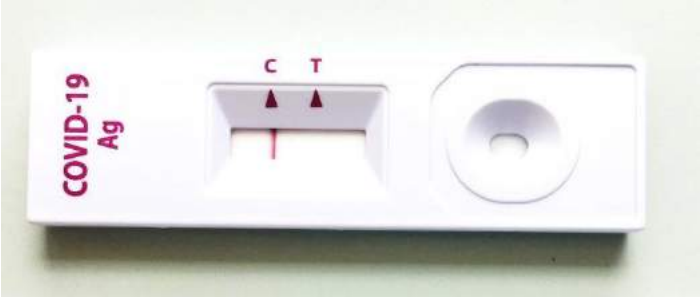
- Complete blood count (CBC), a comprehensive metabolic panel (CMP) that includes renal and liver function testing, and a coagulation panel had to be performed on all hospitalized patients.
- Additional tests, such as ESR, C-reactive protein (CRP), ferritin, lactate dehydrogenase, and procalcitonin, can be considered in hospitalized patients. However, their prognostic significance in COVID-19 is not clear.
- A D-dimer level is required to be guided for the use of therapeutic versus prophylactic doses of anticoagulation.

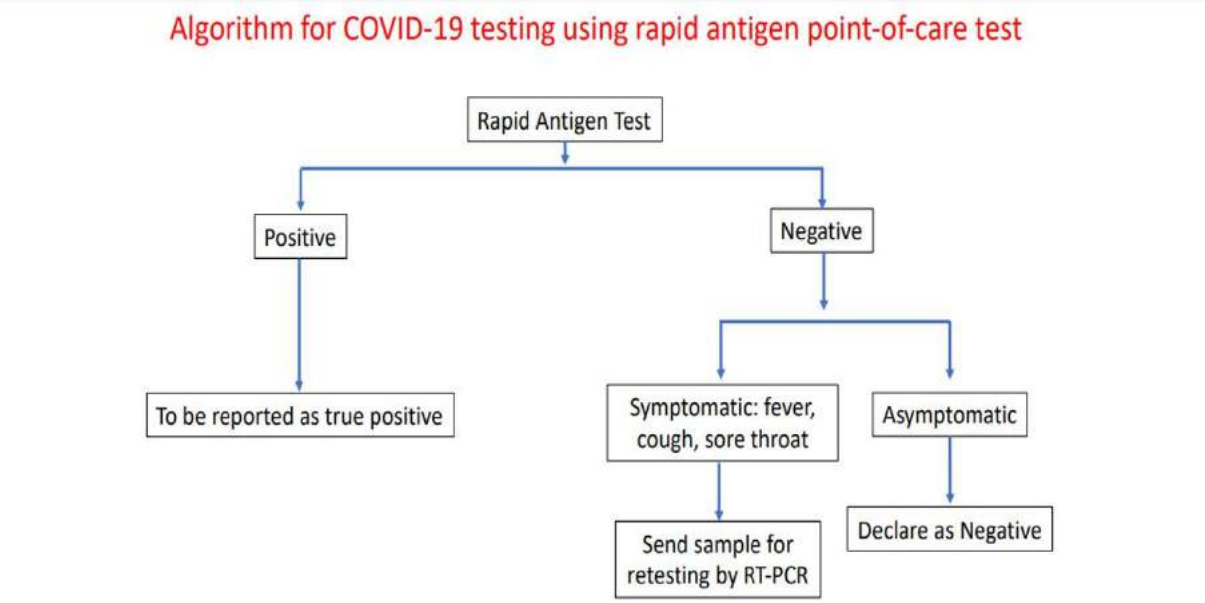
Rapid diagnostic tests based on antigen detection

Rapid diagnostic tests that can be detected the SARS-CoV-2 viral proteins (antigens) is present in respiratory tract of specimen that can bring development and commercialization. Many of them were lateral flow immunoassays (LFI), which can complete typically within 30 minutes. In contrast to NAATs, there were the no amplification in the targeted proteins that can be detected, the antigen tests were becoming less sensitive. Including the false positive (indicating that a person can be infected when they are not) may result that can occur if the antibody that on the test strip that can also identify the antigens of viruses other than SARS-CoV-2, like as human coronaviruses. The sensitivities of different RDTs as

compared to rRT-PCR in specimens from Upper Respiratory Tract (URT) (nasopharyngeal swabs) looks like it is highly variable, but specificity is consistent as reported to be high. Nowdays, data is as the antigen performance is the clinical setting is nowdays still limited: paired NAAT and antigen validations is needed in the clinical studies to encourage & to recongnize that which if the antigen detection tests that can either be performed under the development or that had already been commercialized which can demonstrate acceptable performance in representative field studies. When performance is acceptable, antigen RDTs could be implemented in a diagnostic algorithm to decrease the number of molecular tests that can to be performed and can be supported rapidly by identification and management of COVID-19 cases. How antigen detection could be incorporated with the testing algorithm depends on the sensitivity and specificity of the antigen test and on the prevalence of SARS-CoV-2 infection in the intended testing population. Higher the viral load had to be connected for improved antigen test performance; therefore test performance that is expects had to be best around the symptoms on onset and in the initial phase of a SARS-CoV-2 infection. The antigen(s) detected are expressed only when the virus is actively replicating; therefore, such tests were recommended to identify acute or early infection. The performance of those tests that depends on the time from onset of illness, the concentration of virus seen in the specimen, the quality of the specimen collected from patient and how it is processed.





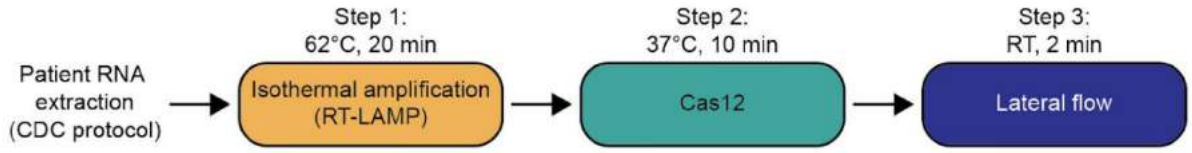


Molecular Assay- Loop-Mediated Isothermal Amplification (LAMP)

Although real-time RT-PCR is sensitive and reliable but it is time-consuming (~2 h) and it needs a specific detection device or instrument, that limit the broad application for current large demands globally in the pandemic of COVID-19. LAMP as it is much more sensitive, easy and also the time efficient method. (LAMP) is a type of rapid technology for DNA amplification which can apply for pathogen detection such as virus, bacteria and malaria. The LAMP reaction generally requires a constant temperature, and also the target DNA can also be amplified in 30 min. There were a COVID-19 diagnosis kit for fast detection of SARS-CoV-2 virus, using one-step reverse transcription and loop-mediated isothermal amplification (RT-LAMP) had been developed. But commercial kits were based on this principle that were not available in India.

Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP)

Recently as the COVID-19 cases were increasing in the world had also encouraged an global effort to develop the point-of-care platforms for diagnosing SARS-CoV-2 cases. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) as it is possible to one of the most promising platforms for rapidly development and accessible SARS-CoV-2 testing cases and had many advantages, compared to RT-qPCR, such as it is highly specific and sensitive, simple operation, rapid amplification, and low cost, RT-LAMP assays had developed for other CoVs cases of the same genus (Beta coronavirus), including SARS-CoV117,118 and MERSCoV. Not surprisingly, many studies had already being demonstrated the uses of RT-LAMP for SARS-CoV-2 detection.



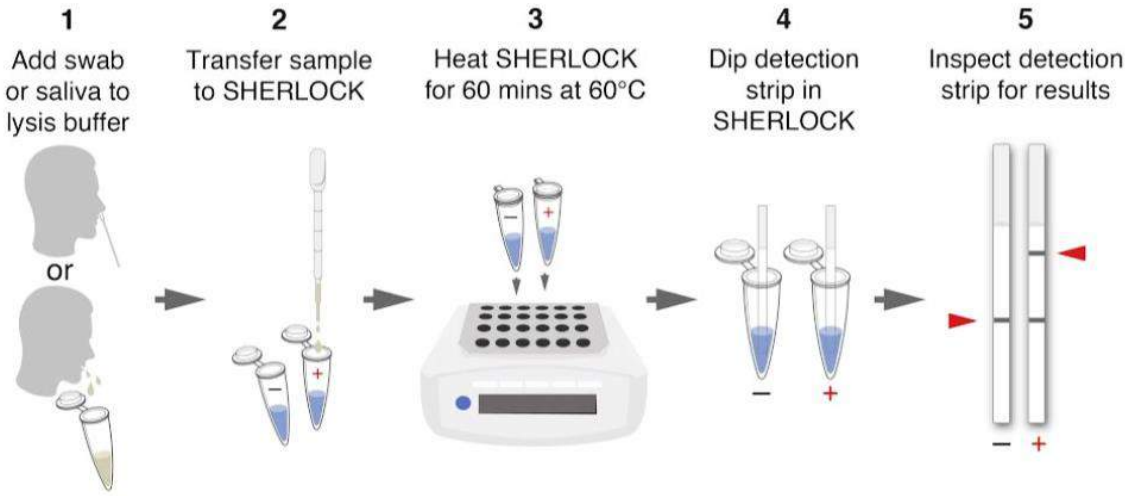
CRISPR/Cas-Based Diagnostic Methods

The cluster of regularly interspaced shortly a palindromic repeats (CRISPR)/Cas machinery had a recent adapted as the POC tool for rapidly detection of nucleic acids (DNA or RNA). Overall, the CRISPR machinery is a program to cleave the specific sequences for the DNA/RNA target that results in an easy observation for combination with a lateral-flow strip.

Initially, Zhang's team develop a CRISPR/Cas-based platform that called it is highly specific and highly sensitive enzymatic reporter unlocking (SHERLOCK) that, combined with isothermal pre-amplification that detect the strains of single-stranded RNA viruses, that identify the mutations of human genotype DNA, and distinguishes the pathogenic bacterias. Most recently, the using of same knowledge, they can adapt a protocol by using the SHERLOCK system for SARS-CoV-2 cases detection.

On the other hand, Mammoth Bioscience Company develop an another platform that was based on the CRISPR/Cas system named the endonuclease-targeted CRISPR trans reporter (DETECTR) to be detected by any other RNA or DNA target, that had now been used to detected with the SARS-CoV-2 RNA genome from the respiratory swab of RNA extracts. As the suitability of DETECTR technology for the identification of SARS-CoV-2 cases were evaluated by using 78 patients specimens, by combining about 36 patients with COVID-19 infection and 42 patients with other viral respiratory infections, and then

compare it with the CDC RTqPCR as a reference method for confirmation of the COVID-19 infection. The SARS-CoV-2 DETECTR test had 95% positive predictive agreement and 100% negative predictive agreement which compare the RT-qPCR results. Despite those promising results, CRISPR/Cas-based diagnostic methods are not now widely used by diagnostic laboratories and need to be the further fulfilment. Taken together, these results highlighted a great potential of CRISPR-based diagnostic methods as rapidly, specificity, portability, and accurately detection of platform by the detection of the SARS-CoV-2 genome in the patient's samples.



Nicking Enzyme-Assisted Reaction (NEAR)

NEAR is a type of driven by two enzymes (nicking endonuclease and DNA polymerase) and with by reaction with buffer, deoxyribonucleotide triphosphate and primers, a linear amplification of DNA template had been achieved. This amplification can eventually leading to be exponentially by increasing the amplified products, and it had to be coupled on a fluorometer.

Nowdays, NEAR reaction that leaded to be at 60 ◦C and it involves five steps: (a) the DNA template is hybridized by primers that were conjugated by nicking endonucleases restriction site, cleaved the double strand DNA (b) from the 30 ends of primer, DNA polymerase increases the nucleotides formation in a double stranded DNA; (c) nicking endonucleases identified the restriction site of the primers and were nicked one of the strands, exposed to the 30 end; (d) DNA polymerase increases from the nicked site by employed uncleaved strands as the template for a new double stranded DNA, displaced the former strands for another cycle of DNA synthesis, but the restriction site was recovered by the newly synthesized double

stranded DNA; (e) Those steps were continuously being amplified targeted DNA template via cleavage, extension and recovery.

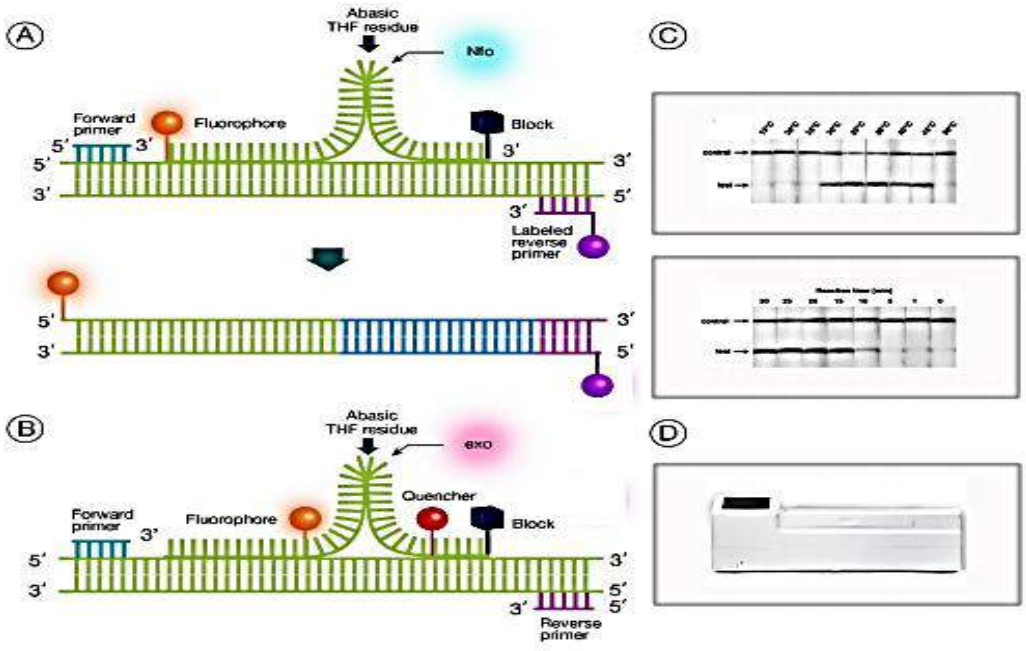


Figure- RPA detection mechanism.

(A) The TwistAmp nfo is a type of lateral flow detection strategy, while by (B) exo probe is a type of real-time detection. The probe that anneals the double stranded DNA has a 30 block (dark blue) that prevents the extension. The Escherichia coli endonuclease IV (nfo) or exonuclease III (exo) that recognizes and cleaves the tetra hydrob furan (THF) residues (as indicated with the arrow) within the probes, detaches the 30 -end block. The process which helps the integration of the amplified products by Bsu polymerase elongation that starts with the 30 -end hydroxide; (A) Regards nfo amplification, fluorophore labels the amplicons (for example, with fluorescein amidites and biotin dyes) that can detects it visually by using lateral flow strips. The sandwich format that allows the fluorophore (bright orange) that captures it by anti-fluorophore conjugated gold nano particles. It can also be identified by a second labelled it by like biotin (purple) that binds to be streptavidin detection line; (B) Regarded to be an exo amplification, fluorescent signals that can generate it when exonuclease III (exo, pink) that can cut the THF site like the nfo, that can also separates the fluorophore (bright orange) from the quencher (red); (C) The lateral flow that can couple it with RPA nfo reaction that could be performed within a broad range of temperatures (top) and also got positive test that can be observed visibly after 10 min (bottom); (D) The

exo fluorescent signals can also be detected with a real-time device, such as the T16-ISO equipment from TwistDx, Cambridge, UK.

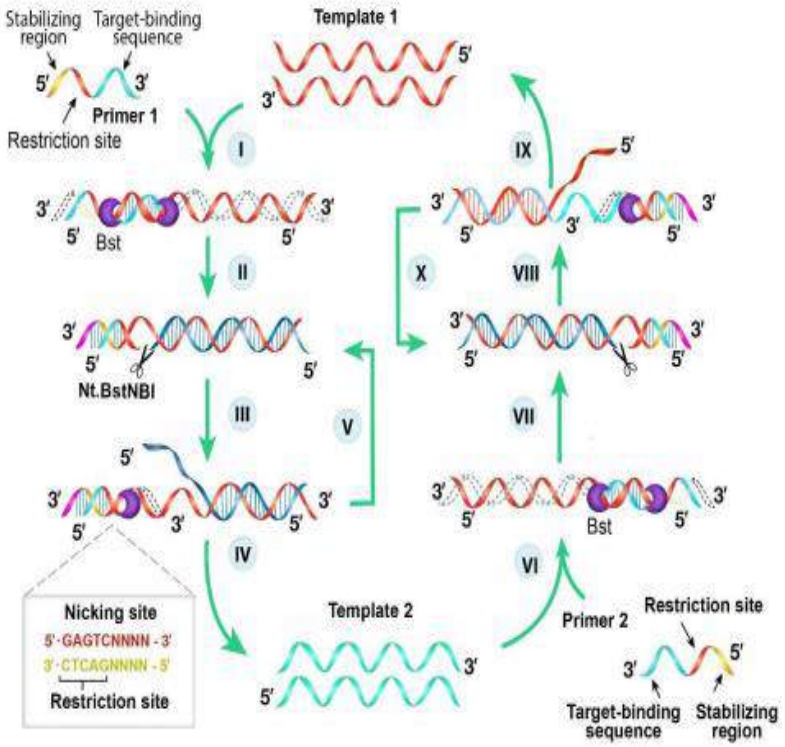


Figure- Schematic representation of nicking enzyme-assisted reaction.

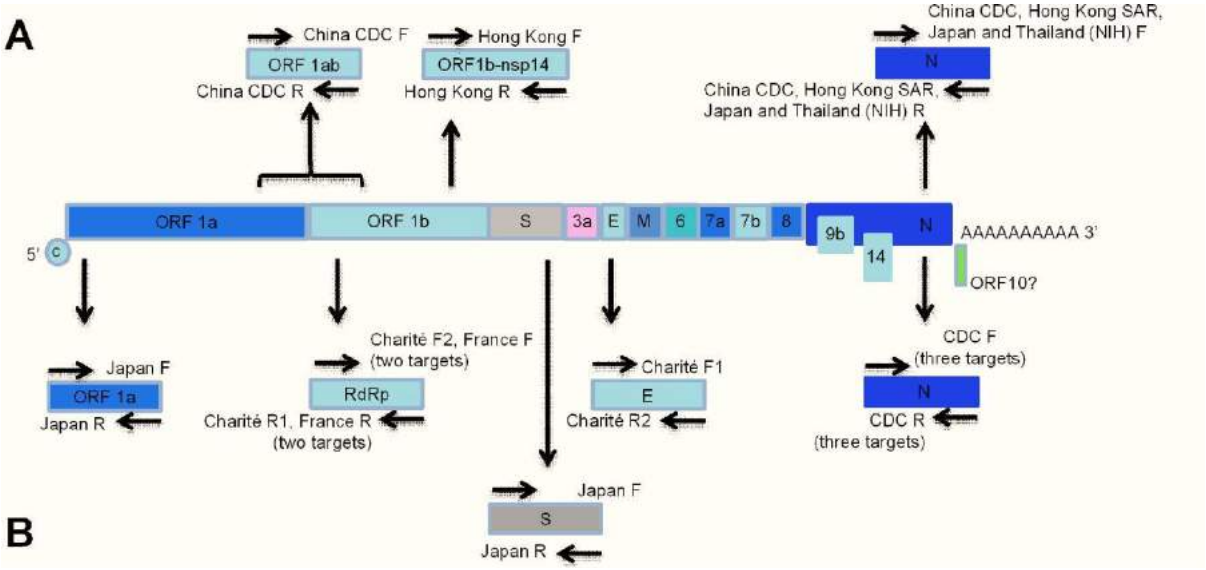
Not alike PCR primers, NEAR primers can be differently designed for successful reaction. Each primer had three regions: restriction site (50 -GAGTCNNNN-30 , for example, Nt. BstNBI), that can stabilize the region and a target-binding region that could be mandatory for the target nucleic acid strand. First of all, the forward primer (primer 1) annealing the template 1 at the target-binding region and can also be elongated by its 30 end by DNA polymerase. That also resulted in formation of an intermediate strand, corelated to template 1 (step I). Simultaneously, the nicking endonuclease recognized the asymmetric restriction site (50 -GAGTC-30) cleaved to strand about four base pairs after the recognizing sequences can also announces a new nick site (step II). At the nick spot, DNA polymerase launches an another extension and eviction is an intermediate strand that can be generated in step I (step III and IV). This eviction strand (template 2) bringing the target-binding sequence accompanied by reverse primer (primer 2), and DNA polymerase can also be elongated from the 30 end of this primer (step VI). Repeatedly, nicking endonuclease identifies and cleaved the restriction site, and the polymerase enzyme elongates and evicts the starting template strand (template 1) from the nick site, which is also be renewed (step VII and

IX) for another cycle, started from step I. The targeted template is exponentially amplified by repetition of cycle for the events.

Genomic sequencing for SARS-CoV-2

Genomic sequencing of SARS-CoV-2 virus can also be recycled to identified for the aggressive for the outbreak, which also includes the size of an epidemic over time, its spatio temporal spread, and testing the hypotheses for transmission routes. On including with the genomic sequences that also be used to get agreed with which diagnostic assays, drugs and vaccines may also be suitable for the patient for further examination. While investigating for SARS-CoV-2 virus genome, accordingly a complement, amplified and support strategies to decrease the disease concern of COVID-19. Nevertheless, the potentiality is high cost and volume of the work needs to be the genomic sequencing which mean that laboratories needed to have precisely about the conventionally the returns from such investment and which is also needed to be maximizing the adequacy of like to be genomic sequencing data.

Whole genomic sequence was also needs to be identified potentially an etiological agents combined to the indexed cases of the COVID-19 patient got pandemic in Wuhan. Involving to be an unequivocally approving the analysis for the SARSCoV-2 infection, regularly the sequence for the percentage of the patient samples while from the clinical cases can also be common to supervise the changes in the viral genome by the time and also the tracing the transmission patterns. For this purpose, many sequencing protocols were also based on Sanger and next-generation sequencing (NGS) were now also being activated for fastly generating with the genomic sequence. SARS-CoV-2 had acquired continuously since it got its emergence. The combing regions for primers and probes need to be supervised continuously by matching the virus genome to be more synchronizing sequences of information that results in more available.



Next generation sequencing (NGS)

The next-generation sequencing (NGS) can also be called to be a high-through put sequence (HTS). By this process we could find the genomic sequence, even more than 1 million base pairs in only one procedure. By this method, we could find the inheritable diseases, cancer, and infectious diseases.

NGS not benefits only in experimentation of novel viral strains on wide scale but also needs a very fast identification for those viruses which can be combine it with human diseases. The NGS technology ahead with bioinformatics tools that were large significance on the modern viral parthenogenesic studies and viral diagnostics. The technology that also plays an important role in the present COVID-19 outbreak. At the beginning of the current outbreak of SARS-CoV2 virus, the samples that were taken from the patients while got admitted for acute respiratory distress syndrome were also be negative for all the suspected already known pathogens and the etiological pathogen were also be identified by only NGS by doing metagenomic RNA sequencing and the phylogenetic analysis of the entire genome generate and also could conclude that it is a new strain of an RNA virus that be a part of the Coronaviridae family and also get designated as SARSCoV2 virus after nucleotide coincidence and genome matting to the existing pathogen's genome. According to this technology there had a large importance for diagnosing the unknown pathogens, and mutation or recombination in the genome for the pathogen in a short interval of time, but at a larger cost of the equipment and chemicals needed in this technique needs to be restricted for utilizing it in daily laboratory analysis of the diseases.

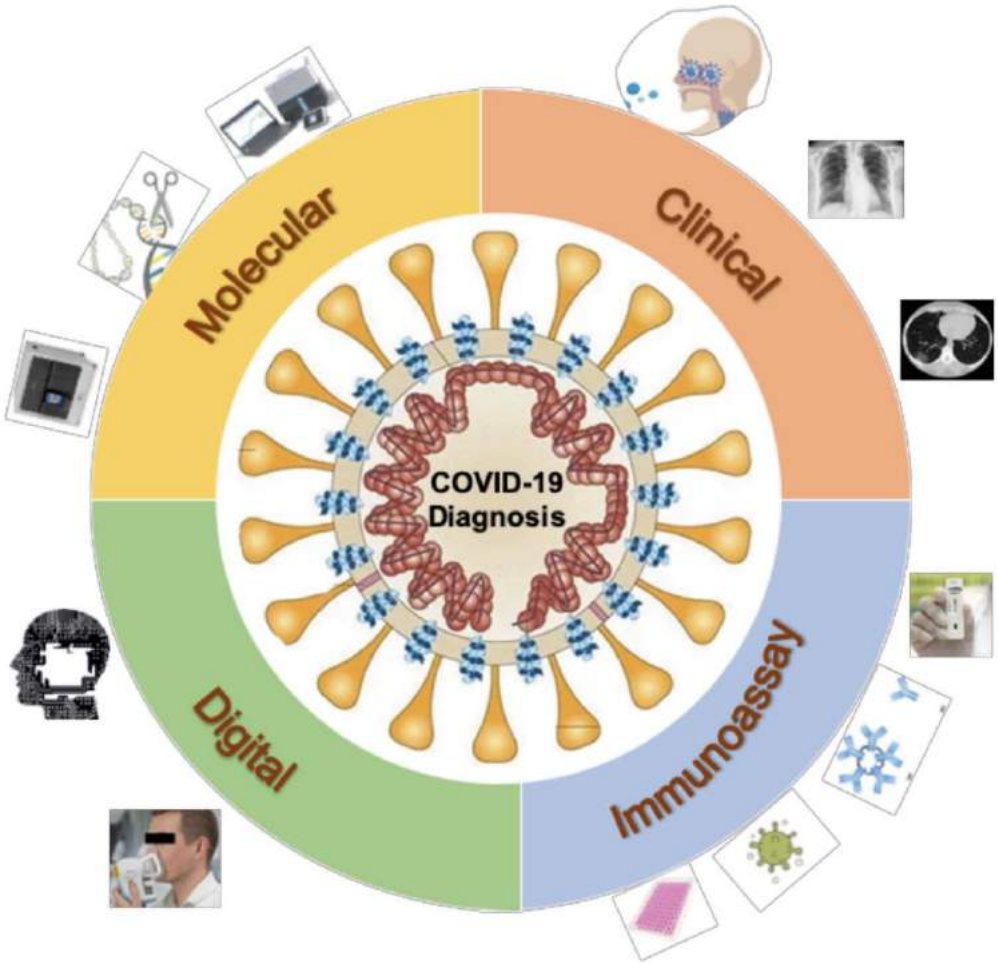
VIRUS CULTURE

The virus culture can also be done by standardizing the methodology as described by other studies briefed about the Vero cells which would be cultured 1 × Dulbecco's modified Eagle's medium (DMEM) in addition to be with 2% fetal bovine serum at 37 ◦C with 5% CO2; need to be inoculation of nasopharyngeal and oropharyngeal samples. And after 3 days of inoculation there were specific cytopathic effects were also be observed. More recently they were also be certified by using real time RT PCR. Researcher from Wuhan, China had done virus isolation in human airway epithelial cells, Vero E6 and Huh-7 cell lines by inoculation of broncho alveolar-lavage samples and can be isolated virus were named 2019-nCoV. When the human airway epithelial cell cultures for virus isolation were skilled labor intensive task, nowadays they were found to be very promising for diagnosing the respiratory pathogens for humans. However some Indian studies reported that the First isolation of SARS-CoV-2 by preparing the use of Vero CCL-81 cells. While some inoculated cells was with nasopharyngeal and oropharyngeal samples, visualized by some specific cytopathic effects for COVID-19 then by these cells that were settle, dehydration and cut into parts for transmission electron microscopy with standard methodology described in some studies. According to a report as they had observed Coronavirus-specific morphology and found that virus particle size ranged from 70 to 90 nm. There were also found to be the virus got resulted in a larger range of intracellular organelles mainly in vesicles. Viral culture of SARS-CoV-2 requires to be managed in a bio-safety Level-3 facility. The cell culture is précised for isolation and characterization of viruses; but it is essential cell culture for virus isolation is not recommend for analysis as purposes.

Table 1 Current Diagnosis method available for COVID-19

Method available	Working principle	Advantage	Time required	Disadvantage
Next generation sequencing (NGS)	Whole genome sequencing	Highly sensitive and specific, Provide all related information; Can identify novel strain	1–2 day	High expertise Equipment dependency and high cost Highly sophisticated Lab required
RT-PCR	Specific primer-probe based detection	Fast results Higher sensitivity Needs small amount of DNA Can be performed in a single step Well established methodology in viral diagnostics	3–4 h	Higher costs due to the use of expensive consumables Expensive lab equipment Detection is also complex and time consuming
LAMP	More than two sets of specific primers pair based detection	Highly repeatable and accurate Single working temperature	1 h	Too sensitive, highly prone to false positives due to carry-over or cross-contamination
Serological (traditional)	Antigen/Antibodies IgG/IgM	Sensitive and specific	4–6 h	Testing come after 3-4 days of infection False positive
Rapid serological	Antigen/Antibodies IgG/IgM	POCT	15–30 min	Testing come after 3-4 days of infection False positive
CT scan	Chest images	Enhance sensitivity of detection if findings combined with RT-PCR results	1 h	Indistinguishability from other viral pneumonia and the hysteresis of abnormal CT
Virus isolation	In vitro live virus isolation and propagation	Highly (100%) specific Gold standard	5–15 days	Low sensitivity as isolation is not 100%


31st Dec 2019: China reports atypical pneumonia in Wuhan to WHO
11th Jan 2020: First death in confirmed positive patient
12th Jan 2020: Genetic sequence of SARS CoV 2 revealed
13th Jan 2020 First case outside China in Thailand
30th Jan 2020 WHO declares COVID-19, an international public health emergency
2nd Feb 2020 First Death outside China in Phillippines
11th March 2020: WHO declared COVID-19 as a pandemic
30 April 2020: More than 3 million cases and 0.2 million deaths across 210 countries


Molecular
Clinical
Digital
Immunoassay
COVID-19
Diagnosis

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