VARIOUS DIAGNOSTIC APPROACHES FOR DETECTION OF SARS-CoV-2 INFECTION

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INTRODUCTION

This chapter provides useful guidance to laboratories and other stakeholders involved in diagnostics for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

It mainly covers the various aspects of specimen collection, nucleic acid amplification testing (NAAT), antigen (Ag), antibody (Ab) detection and quality assurance.

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 first alerted to a cluster of pneumonia of unknown etiology in Wuhan, People's Republic of China on 31 December 2019. The virus was initially tentatively named 2019 novel coronavirus (2019-nCoV).

Subsequently the International Committee of Taxonomy of Viruses (ICTV) named the virus SARS-CoV-2. COVID-19 is the name of the illness caused by SARS-CoV-2.

The SARS-CoV-2 is classified within the genus *Betacoronavirus* (subgenus *Sarbecovirus*) of the family *Coronaviridae*. This is an enveloped, positive sense, single-stranded ribonucleic acid (RNA) virus with a 30-kb genome. The virus has an RNA proofreading mechanism keeping the mutation rate relatively low.

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

The SARS-CoV-2 is the seventh coronavirus identified that is known to infect humans (HCoV). Four of these viruses, HCoV-229E, HCoV-NL63, HCoV-HKU1 and HCoV-OC43, are 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 these viruses belong to the subgenus *Sarbecovirus* within the genus *Betacoronavirus*.

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

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

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

The diagnostic testing chiefly depends upon 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).

At the global level, sufficient testing capacity for COVID-19 is not available as it should be and therefore preventing 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 2020d).

Therefore, early diagnosis and prompt treatment can substantially reduce 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 are in close contact with suspicious exposure have been advised under a 14-day health observation period that should be started from the last day of contact with infected individuals.

Once these individuals show any symptoms including coughing, sneezing, shortness of breath or diarrhoea, they should require immediate medical attention. Immediate isolation of the suspected individual should be performed 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 performed for those who were in contact with the suspected or confirmed individuals by observing their clinical symptoms. Before taking decision about isolation, authorities should make sure that whether the suspected individual requires home isolation and careful clinical evaluation with safety assessment by healthcare professionals or not.

If the suspected individuals present with any symptoms during isolation, they should immediately contact the doctors for their treatment.

During home isolation, suggested medication and symptoms should be closely recorded.

The suspected, probable and confirmed case definition of COVID-19 by the WHO has been presented in Fig. 9.1.

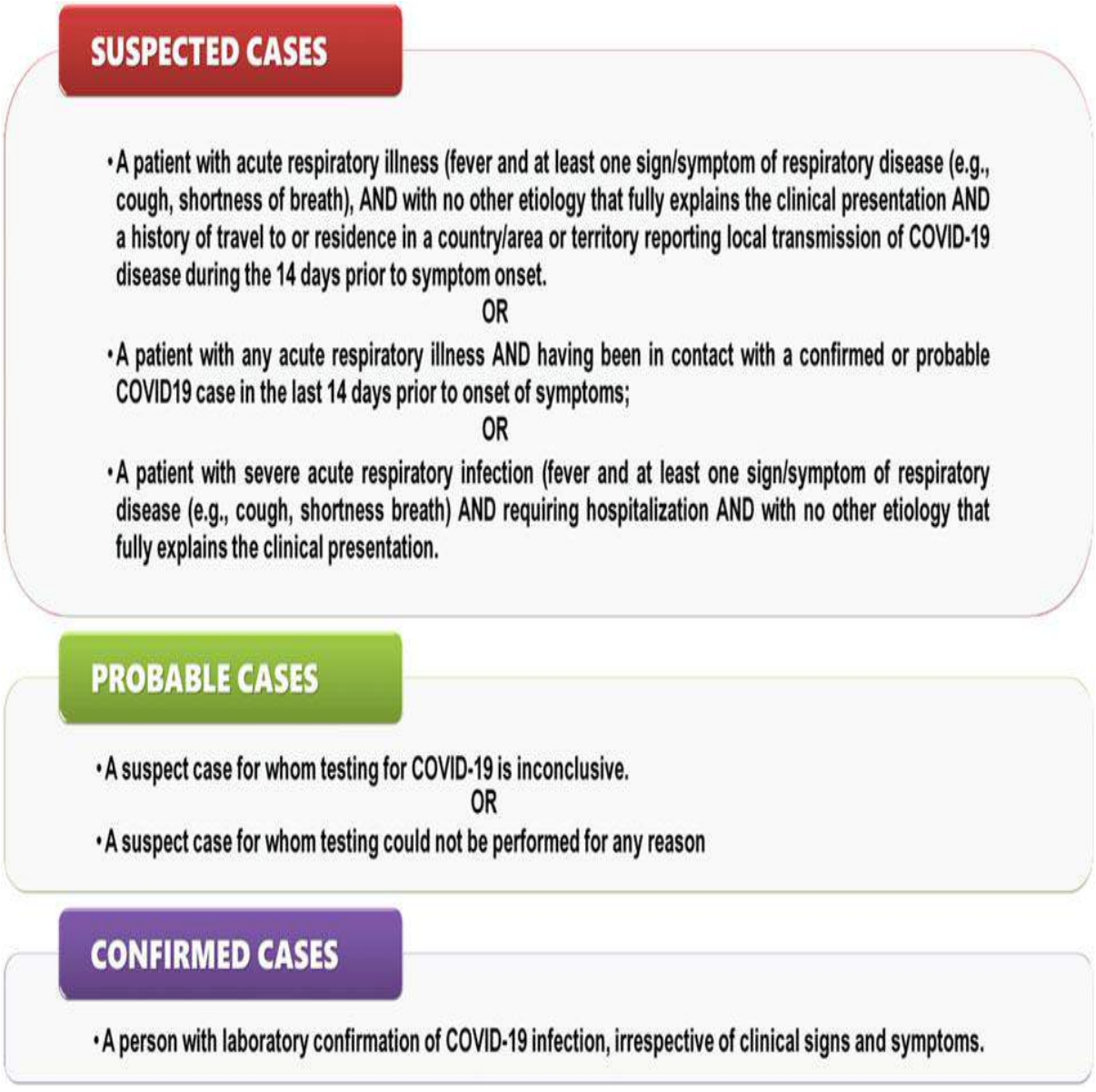


Fig. 9.1 Case definition of COVID-19 by the World Health Organization.

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

Laboratory virology tests are essential for a correct diagnosis and the population level prevalence of COVID-19, given the number of asymptomatic cases or nonspecific clinical symptoms. Results from these tests guide clinicians and health officials in the management, control, and prevention of COVID-19.

There are principally two types of tests available for COVID-19: viral tests and antibody tests. The viral tests are direct tests as they are designed to detect the virus and therefore reflect current infection. In contrast, the antibody tests are indirect tests, as they do not detect the virus, but rather ascertain established seroconversion to previous infection, or early seroconversion to ongoing infection.

Direct- The recommended test for diagnosis of SARS-CoV-2 infection involves detection of viral RNA using nucleic acid amplification tests (NAAT), such as reverse transcription (RT)-PCR; Antigen detection tests are designed to directly detect viral particles in biological samples such as nasopharyngeal secretions.

- **Indirect-** In contrast to NAAT-based testing, where as soon as the sequence is known, a diagnostic test can be built, the diagnostic technology and methodology underlying the development of serological tests is quite different, with a substantially longer timeline to obtain a robust product that is suitable for routine 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

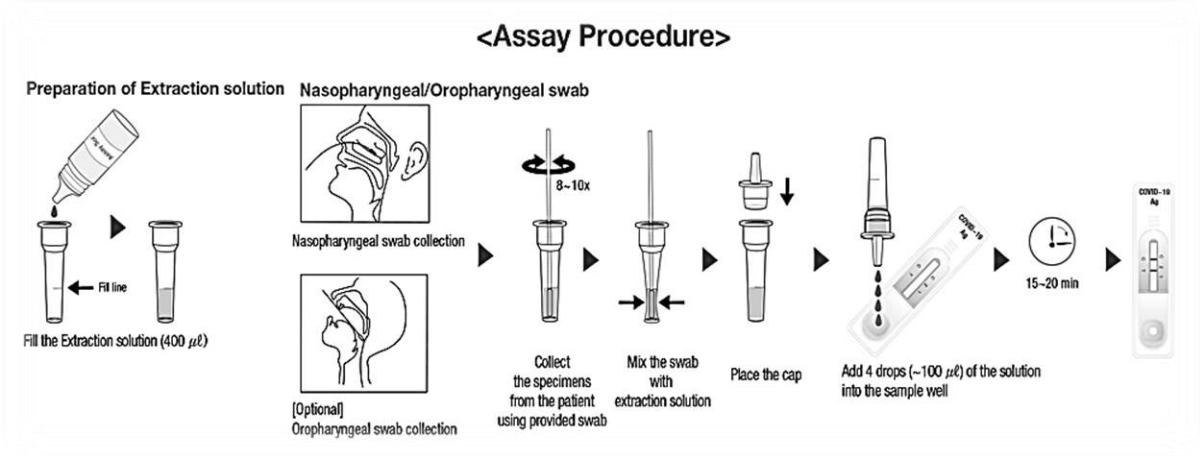
Indirect-

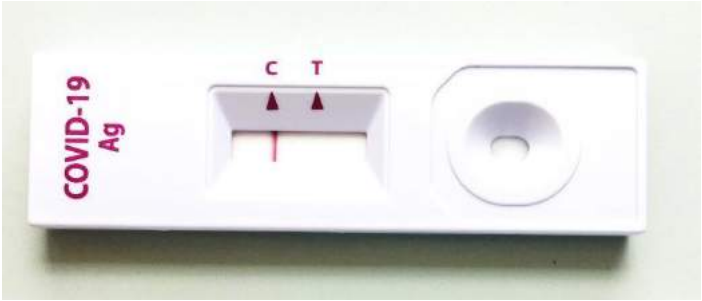
- **Detection of specific antibodies**
- Enzyme-linked immunosorbent assays (ELISA)
- Immunofluorescence assays (IFA)
- Lateral flow assays (LFA)
- Chemiluminescence enzyme immunoassays (CLIA)
- Magnetic chemiluminescence enzyme immunoassay (MCLIA)

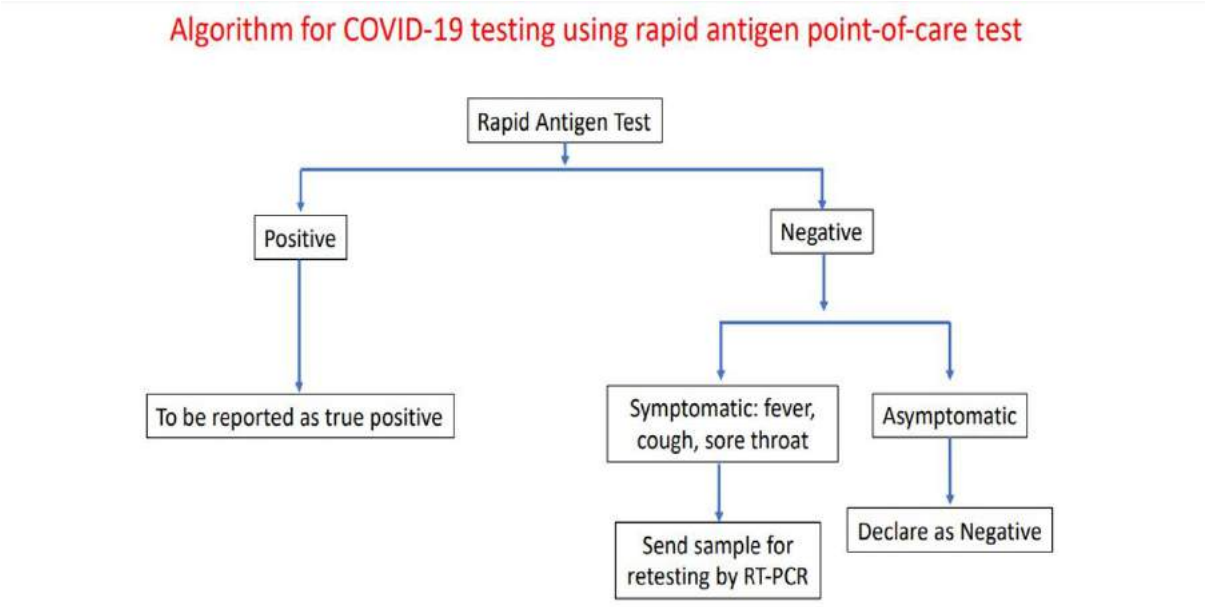
Rapid diagnostic tests based on antigen detection

Rapid diagnostic tests that detect the presence of SARS-CoV-2 viral proteins (antigens) in respiratory tract specimens are being developed and commercialized. Most of these are lateral flow immunoassays (LFI), which are typically completed within 30 minutes. In contrast to NAATs, there is no amplification of the target that is detected, making antigen tests less sensitive. Additionally, false positive (indicating that a person is infected when they are not) results may occur if the antibodies on the test strip also recognize antigens of viruses other than SARS-CoV-2, such other human coronaviruses. The sensitivity of different RDTs compared to rRT-PCR in specimens from URT (nasopharyngeal swabs) appears to be highly variable, but specificity is consistently reported to be high. Currently, data on antigen performance in the clinical setting is still limited: paired NAAT and antigen validations in clinical studies are encouraged to identify which of the antigen detection tests that are either under development or

have already been commercialized demonstrate acceptable performance in representative field studies. When performance is acceptable, antigen RDTs could be implemented in a diagnostic algorithm to reduce the number of molecular tests that need to be performed and to support rapid identification and management of COVID-19 cases. How antigen detection would be incorporated into 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 viral loads are associated with improved antigen test performance; therefore test performance is expected to be best around symptom 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 are recommended to identify acute or early infection. The performance of these tests depends on the time from onset of illness, the concentration of virus in the specimen, the quality of the specimen collected from a person and how it is processed.







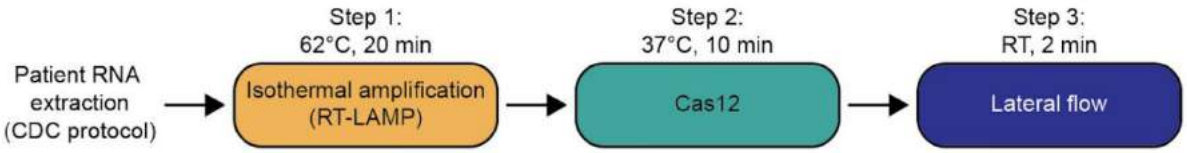
Molecular Assay- Loop-Mediated Isothermal Amplification (LAMP)

Although real-time RT-PCR is sensitive and reliable, it is time-consuming (~2 h) and requires a specific detection device or instrument, which limits its broad application to current huge demand for the global pandemic of COVID19. LAMP It is a very sensitive, easy and time efficient method. (LAMP) is a rapid technology of DNA amplification which has been applied to pathogen detection such as virus, bacteria and malaria. The LAMP reaction generally needs one constant temperature, and the target DNA can be amplified in 30 min. COVID-19 diagnosis kit for the rapid detection of SARS-CoV-2, using one-step reverse transcription and loop-mediated isothermal amplification (RT-LAMP) has been developed. But commercial kits based on this principle are not yet available in India.

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

The recent increase in the number of COVID-19 cases in the world has encouraged a global effort to develop point-of-care platforms for diagnosing SARS-CoV-2. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) is perhaps one of the most promising platforms for rapid development and accessible SARS-CoV-2 testing and has many advantages, compared to RT-qPCR, such as high specificity and sensitivity, simple operation, rapid amplification, and low cost, RT-LAMP assays have been developed for other CoVs of the same genus (Beta coronavirus), including SARS-CoV117,118

and MERSCoV. Not surprisingly, several studies have already demonstrated the use of RT-LAMP for SARS-CoV-2 detection.

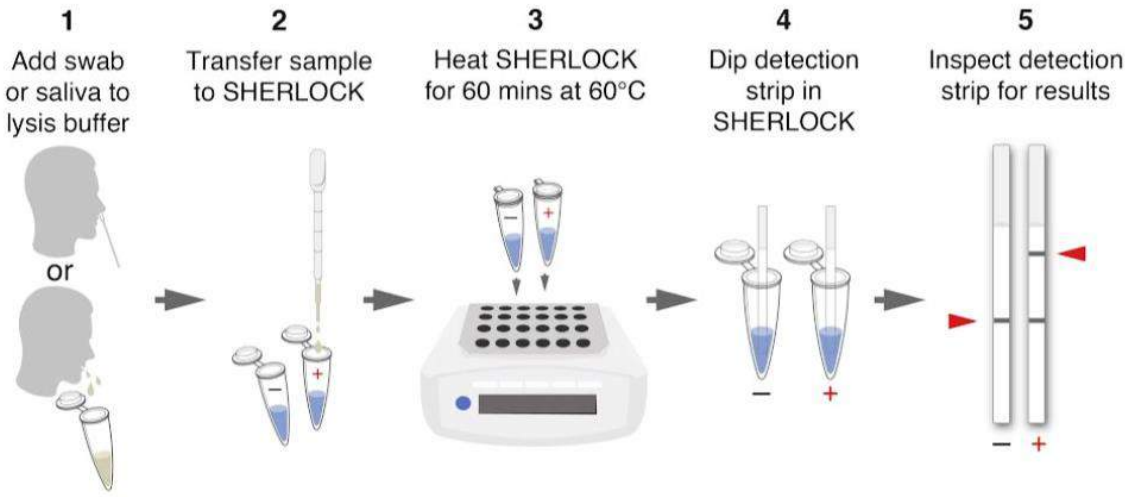


CRISPR/Cas-Based Diagnostic Methods

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas machinery has recently been adapted as a POC tool for the rapid detection of nucleic acids (DNA or RNA). Overall, this CRISPR machinery is programmed to cleave specific sequences in the DNA/RNA target where the results can be easily observed by combination with a lateral-flow strip.

Initially, Zhang's team developed a CRISPR/Cas-based platform called specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) that, combined with isothermal preamplification to detect strains of single-strand RNA viruses, identifies mutations and human genotype DNA, and distinguishes pathogenic bacteria. More recently, using the same knowledge, they adapted a protocol using the SHERLOCK system for SARS-CoV-2 detection.

On the other hand, Mammoth Bioscience Company developed another platform based on the CRISPR/Cas system named the endonuclease-targeted CRISPR trans reporter (DETECTR) to detect any RNA or DNA target, which has now been used to detect the SARS-CoV-2 RNA genome from respiratory swab RNA extracts. The suitability of DETECTR technology for the detection of SARS-CoV-2 was evaluated using 78 patient specimens, including 36 patients with COVID-19 infection and 42 patients with other viral respiratory infections, and then compared with the CDC RTqPCR as a reference method to confirm COVID-19 infection. The SARS-CoV-2 DETECTR test had 95% positive predictive agreement and 100% negative predictive agreement when compared with RT-qPCR results. Despite these promising results, CRISPR/Cas-based diagnostic methods are not widely used by diagnostic laboratories and need further implementation. Taken together, these results highlight the great potential of CRISPR-based diagnostic methods as a rapid, specific, portable, and accurate detection platform for the detection of the SARS-CoV-2 genome in patient samples.



Nicking Enzyme-Assisted Reaction (NEAR)

NEAR is driven by two enzymes (nicking endonuclease and DNA polymerase) and with reaction buffer, deoxyribonucleotide triphosphate and primers, a linear amplification of DNA template is achieved. This amplification eventually leads to exponential increase of amplified products, and it can be coupled on a fluorometer.

Briefly, NEAR reaction occurs at 60 ◦C and it involves five steps: (a) the DNA template is hybridized with primers that are conjugated with nicking endonucleases restriction site, cleaving the double stranded DNA (b) from the 30 end of the primer, DNA polymerase extends nucleotides forming a double stranded DNA; (c) nicking endonucleases identifies the restriction site on the primers and nicked one of the strands, exposing the 30 end; (d) DNA polymerase extends from the nicked site by employing uncleaved strand as a template for a new double stranded DNA, displacing the former strand for another cycle of DNA synthesis, but restriction site is recovered on the newly synthesized double stranded DNA; (e) These steps continuously amplified targeted DNA template via cleavage, extension and recovery.

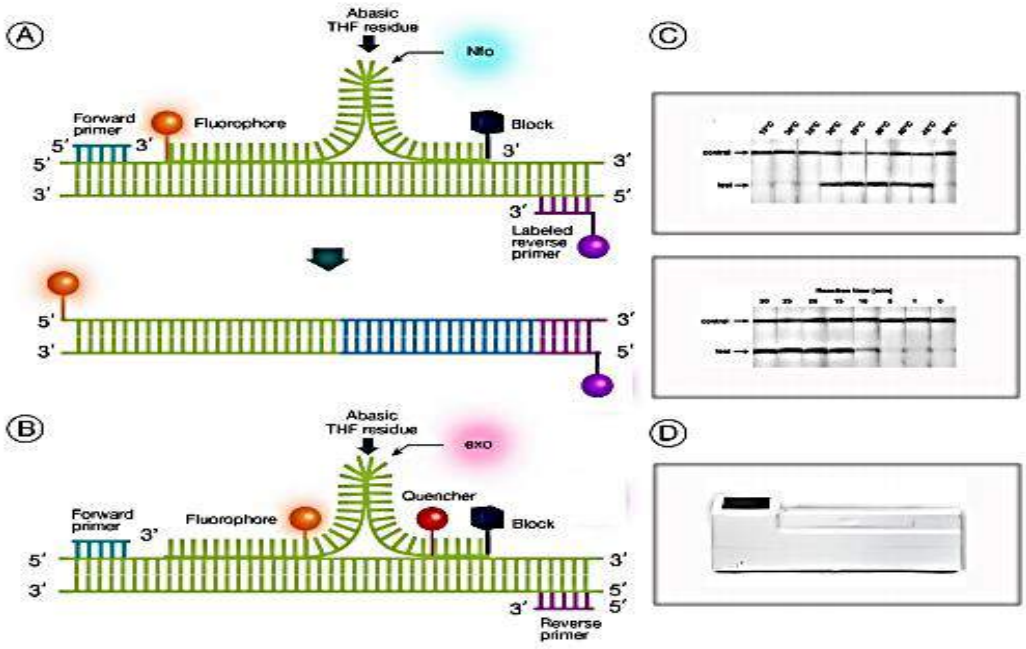


Figure- RPA detection mechanism.

(A) The TwistAmp nfo is for lateral flow detection strategy, while (B) exo probe is for real-time detection. The probe annealed to double stranded DNA has a 30 block (dark blue) that prevents extension. The Escherichia coli endonuclease IV (nfo) or exonuclease III (exo) recognizes and cleaves the tetrahydrofuran (THF) residue (as indicated with the arrow) within the probe, detaching the 30 -end block. This process helps the integration into the amplified products through Bsu polymerase elongation from the 30 -end hydroxide; (A) Regarding nfo amplification, fluorophore labeled amplicons (for example, with fluorescein amidites and biotin dyes) can be detected visually using lateral flow strips. This sandwich format allows the fluorophore (bright orange) to be captured through anti-fluorophore conjugated gold nanoparticles. It also can detect a second label like biotin (purple) by binding to a streptavidin detection line; (B) Regarding exo amplification, fluorescent signals are generated when exonuclease III (exo, pink) cuts the THF site like the nfo, separating the fluorophore (bright orange) from the quencher (red); (C) The lateral flow coupled with RPA nfo reaction can be performed within a broad range of temperatures (top) and a positive test is observed visibly after 10 min (bottom) [54]; (D) The exo fluorescent signals are detected by a real-time device, such as the T16-ISO equipment from TwistDx, Cambridge, UK.

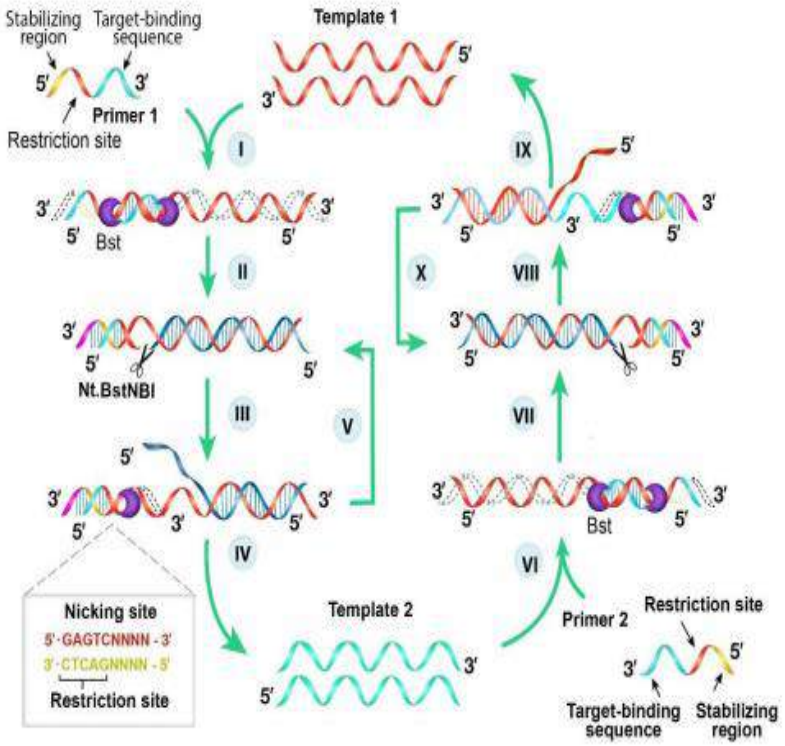


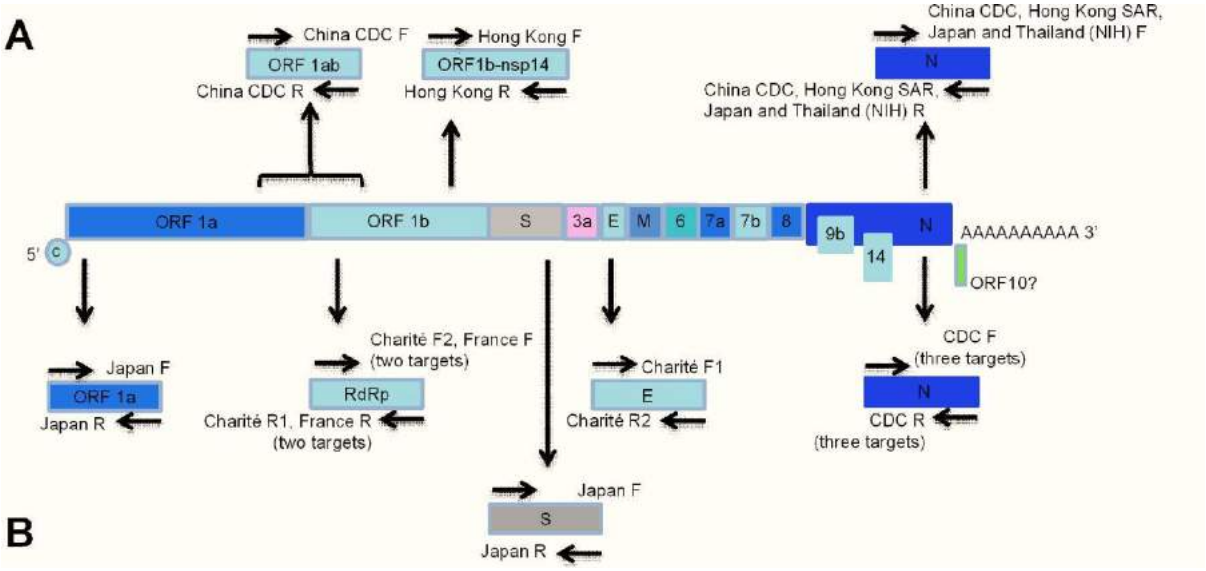
Figure- Schematic representation of nicking enzyme-assisted reaction.

Unlike PCR primers, NEAR primers are uniquely designed for a successful reaction. Each primer has three regions: restriction site (50 -GAGTCNNNN-30 , for example, Nt. BstNBI), stabilizing region and a target-binding region that is complementary to the target nucleic acid strand. First, the forward primer (primer 1) anneals with template 1 at the target-binding region and extended from its 30 end by DNA polymerase. This results in the formation of an intermediate strand, complementary to template 1 (step I). At the same time, the nicking endonuclease recognizes the asymmetric restriction site (50 -GAGTC-30) cleaves the strand with four base pairs after the recognized sequence introduces a new nick site (step II). At the nick site, DNA polymerase initiates another extension and displace the intermediate strand generated in step I (step III and IV). This displaced strand (template 2) carries the target-binding sequence complementary to reverse primer (primer 2), and DNA polymerase extends from the 30 end of this primer (step VI). Again, nicking endonuclease recognizes and cleaves the restriction site, and the polymerase enzyme extends and displaces the initial template strand (template 1) from the nick site, which is recovered (step VII and IX) for another cycle, starting from step I. The target template is exponentially amplified by repeating this cycle of events.

Genomic sequencing for SARS-CoV-2

Genomic sequencing for SARS-CoV-2 can be used to investigate the dynamics of the outbreak, including changes in the size of an epidemic over time, its spatiotemporal spread, and testing hypotheses about transmission routes. In addition, genomic sequences can be used to decide which diagnostic assays, drugs and vaccines may be suitable candidates for further exploration. Analysis of SARS-CoV-2 virus genomes can, therefore, complement, augment and support strategies to reduce the disease burden of COVID19. However, the potentially high cost and volume of the work required for genomic sequencing means that laboratories should have clarity about the expected returns from such investment and what is required to maximize the utility of such genomic sequence data.

Whole genome sequencing was used to identify potential etiological agents involved in the index cases of the COVID-19 pandemic in Wuhan. In addition to unequivocally confirming the diagnosis of a SARSCoV-2 infection, regular sequencing of a percentage of patient samples from clinical cases can be used to monitor changes in the viral genome over time and trace transmission patterns. For this purpose, several sequencing protocols based on Sanger and next-generation sequencing (NGS) are being applied to rapidly generate the genome sequences. SARS-CoV-2 has evolved continuously since its emergence. The binding regions of primers and probes should be monitored continuously for matching to the virus genome as more sequence information becomes available.



Next generation sequencing (NGS)

The, next-generation sequencing (NGS) is also called as high-throughput sequencing (HTS). By this method we can determine the genomic sequence, even more than 1 million base pairs in a single experiment. By this technique, we can diagnose the inheritable diseases, cancer, and infectious diseases.

NGS helps not only in discovery of novel viral strains on large scale but also provides very rapid detection of these viruses which link with human diseases. The NGS technology along with bioinformatics tools have largely influenced the modern viral parthenogenesis studies and viral diagnostics. This technology also played great application in the present COVID-19 outbreak. At initiation of current outbreak of SARS-CoV2, the samples from the patients admitted acute respiratory distress syndrome were negative for the all suspected already known pathogens, the etiological pathogen was identified by only NGS by doing metagenomic RNA sequencing and the phylogenetic analysis of its complete genome generated could conclude that it is a new strain of an RNA virus which belonged to the Coronaviridae family and was designated as SARSCoV2 after nucleotide similarity and genome matching with the existing pathogen's genome [33].Therefore, this technology has great importance for identifying unknown pathogens, and mutation or recombination in the genome of the pathogen in a short span of time, but the huge cost of the equipment and chemicals required in this technique restricts its utilization in routine laboratory diagnosis of the diseases

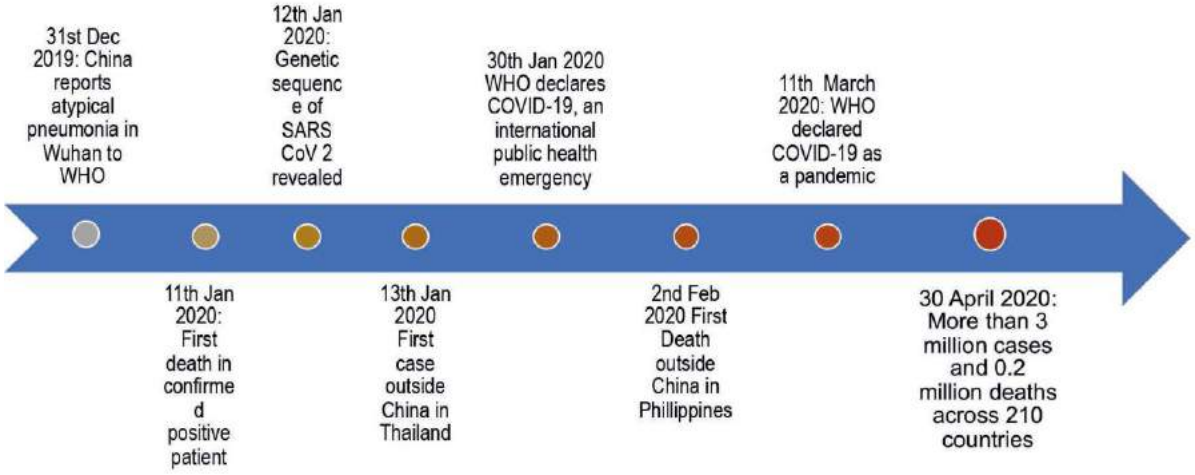
VIRUS CULTURE

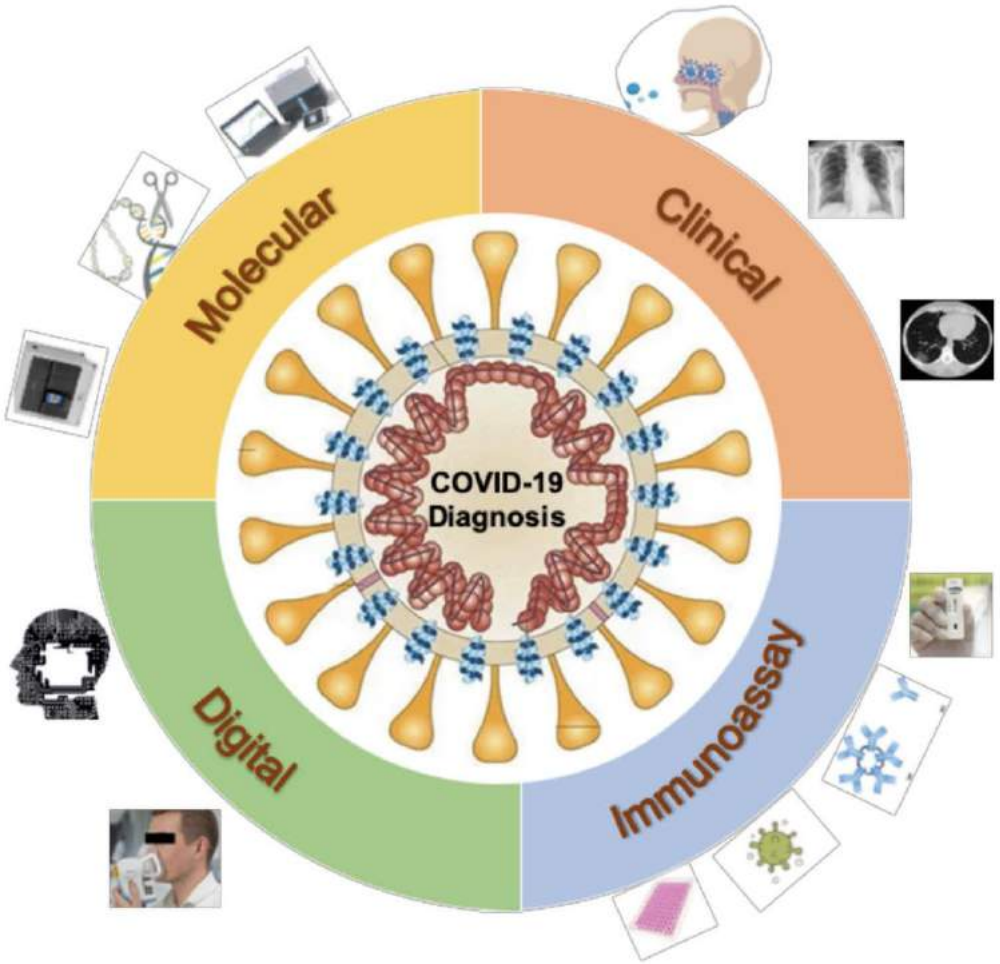
The virus culture can be done by standard methodology as described by Kim et al; briefly the Vero cells which were cultured 1 × Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum at 37 ◦C with 5% CO2; used for the inoculation of nasopharyngeal and oropharyngeal samples. And after 3 days of inoculation the specific cytopathic effects were observed. Later on they are also confirmed by using real time RT PCR. Researcher from Wuhan, China have done virus isolation on human airway epithelial cells and Vero E6 and Huh-7 cell lines by inoculation of bronchoalveolar-lavage samples and the isolated virus was named 2019-nCoV. Using the human airway epithelial cell cultures for virus isolation is skilled labor intensive task, however these were found very promising for analysis of respiratory pathogens of humans. Recently an Indian study reported the First isolation of SARS-CoV-2 by using Vero CCL-81 cells. The inoculated cells with nasopharyngeal and oropharyngeal samples, visualized for specific cytopathic effects for COVID-19 then these cells were fixed, dehydrated and cut into sections for transmission electron microscopy with standard methodology described by Kim et al.

Kim et al reported that they have observed Coronavirus-specific morphology and found the virus particle size ranged from 70 to 90 nm. They also found that the virus is observed in wide range of intracellular organelles especially in vesicles [18]. Viral culture of SARS-CoV-2 needs to be conducted in a bio-safety Level-3 facility. The cell culture is very useful for isolation and characterization of viruses; but basically the cell culture for virus isolation is not recommended for diagnostic 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



REFERENCE

1) Diagnostic testing for SARS-CoV-2, Interim guidance 11 September 2020 WHO
2) Chapter 9 : Laboratory Diagnosis of Novel Coronavirus Disease 2019 COVID-19 Infection A. Padhi, E. Gupta, S.K. Saxena (ed.), Coronavirus Disease 2019 (COVID-19) Medical Virology: from Pathogenesis to Disease Control, https://doi.org/10.1007/978-981-15-4814-7_9
3) ACS Infect. Dis. 2020, 6, 9, 2319–2336; Publication Date: August 4, 2020; https://doi.org/10.1021/acsinfecdis.0c00274
4) La Marca A, Capuzzo M, Paglia T, Roli L, Trenti T, Nelson SM. Testing for SARS-CoV-2 (COVID-19): a systematic review and clinical guide to molecular and serological in-vitro diagnostic assays. Reprod Biomed Online. 2020;41(3):483-499. doi:10.1016/j.rbmo.2020.06.001

5) La Marca A, Capuzzo M, Paglia T, Roli L, Trenti T, Nelson SM. Testing for SARS-CoV-2 (COVID-19): a systematic review and clinical guide to molecular and serological in-vitro diagnostic assays. Reprod Biomed Online. 2020;41(3):483-499. doi:10.1016/j.rbmo.2020.06.001.
6) Clinical and Laboratory Diagnosis of SARS-CoV-2, the Virus Causing COVID-19 Severino Jefferson Ribeiro da Silva, Caroline Targino Alves da Silva, Klarissa Miranda Guarines, Renata Pessôa Germano Mendes, Keith Pardee, Alain Kohl, and Lindomar Pena;*ACS Infectious Diseases* **2020** *6* (9), 2319-2336 DOI: 10.1021/acsinfecdis.0c00274.
7) COVID-19 Infection Diagnosis: Potential Impact of Isothermal Amplification Technology to Reduce Community Transmission of SARS-CoV-2 Ameh S. James * and John I. Alwneh.
8) VirusDis. (April–June 2020) 31(2):97–105 https://doi.org/10.1007/s13337-020-00599-7.
9) Laboratory diagnosis of SARS-CoV-2 - A review of current methods Jitendra Prasad Mathuriaa, Ramakant Yadav b, Rajkumar c.