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REVIEW ARTICLE

Diagnostic Approaches for Coronavirus Disease 2019 (COVID-19)

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ABSTRACT

On December 31, 2019, China reported an epidemic result of simply pneumonia. The Chinese government identified the virus (2019-nCoV) and declared an epidemic as this results in thousands of positive human infections in many countries like China, USA. The emission of the Severe Acute Respiratory Syndrome (SARS) in 2002/2003 has indicated the possibility of transmission from animal-to-human and human-to-human. The spasmodic emergence and the eruption of various types of CoVs apprise us that CoVs is a severe global health threat. Thus, there is an urgent need to develop effective vaccines against CoVs to just penalize this critical situation. Here, in this paper, we have discussed about the different approaches for the diagnosis of COVID-19.

Keywords: SARS, CoVs, Antibody, Diagnosis, RT-PCR.

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INTRODUCTION

In 1898, Beijerinck [6] discovered 'virus' through the filtrate of infected leaves as in 1892, Ivanovski [28] demonstrated the presence of some of the infectious agent. Tobacco mosaic virus (TMV) was the first discovered virus.

Viruses are the smallest among all the microorganisms. A virus is an infectious agent that can only replicate within the host. They depend on the host cells of organisms. They are made up of genetic core material, either DNA or RNA surrounded by a protective proteinaceous layer called 'Capsid'. Sometimes, there is a presence of spikes on the coat called 'Envelope'. A Virion consists of the nucleic acid and protein layer. They encode 4 – 200 proteins [29].

CORONA VIRUS- AN EPIDEMIC DISEASE

On December 31, 2019, China reported an epidemic result of simply pneumonia. The Chinese government identified the virus (2019-nCoV) and declared an epidemic as this results in thousands of positive human infections in many countries like China, USA. The name 'Coronavirus' derived from the Latin word corona means 'crown' discovered by June Almeida and David Tyrell(1966). They can infect the gastrointestinal, hepatic, respiratory and central nervous system of humans, birds, mouse, livestock, bat and many other wild animals [(10)]. This virus is evolving since 1930 (Table-1). The emission of the Severe Acute Respiratory Syndrome (SARS) in 2002/2003 has indicated the possibility of transmission from animal-to-human and human-to-human. The spasmodic emergence and the eruption of various types of CoVs apprise us that CoVs are severe global health threat [12]. Thus, there is an acute need to develop effectual vaccines against CoVs to just penalize this critical situation [21, 43].

Around 181 reported data, symptoms developed in about 2.5% of infected ones within 2.2 days and 97.5% of others infected in 11.5 days [31]. The estimated incubation period was 5.1 days. The Chinese Center for Disease Control and Prevention reported approximately 44,500 confirmed cases with an estimation of the seriousness of disease [44, 11]. Symptoms like mild were reported in 81% of the population, severe disease (dyspnea, hypoxia, or >50 % involvement of lung on imaging within 24-48 hours) was reported in 14 %, critical disease (respiratory failure, shock) was reported in 5% and no deaths occurred in non-critical cases [22, 13]. Among the 44,500 confirmed cases, 87% of patients were between 30 and 79 years old, the hospitalization rate increased with age includes 1% for 20 to 29 years old, 4% for 50-59 years old and 18% for older than 80 years. Approximately, 1014 patients in China diagnosed under both RT-PCR testing and medical imaging for the detection of COVID-19, a "positive" result shows a sensitivity of 97%, through PCR test [3]. COVID-19 cases make show peripheral distribution (80% v/s 57%), ground-glass opacities (91% v/s 68%), fine reticular opacities (56% v/s 22 %), thickening of vascular (59% v/s 22 %) and reverse halo sign (11% v/s 1%), but mostly it shows central and peripheral distribution (14% v/s 35%), air bronchogram (14% v/s 23 %), pleural thickening (15% v/s 33 %), pleural effusion (4% v/s 33 %) and lymphadenopathy (2.7% v/s 10%) [4]. Major radiologists differentiate COVID-19 according to high specificity and moderate sensitivity.

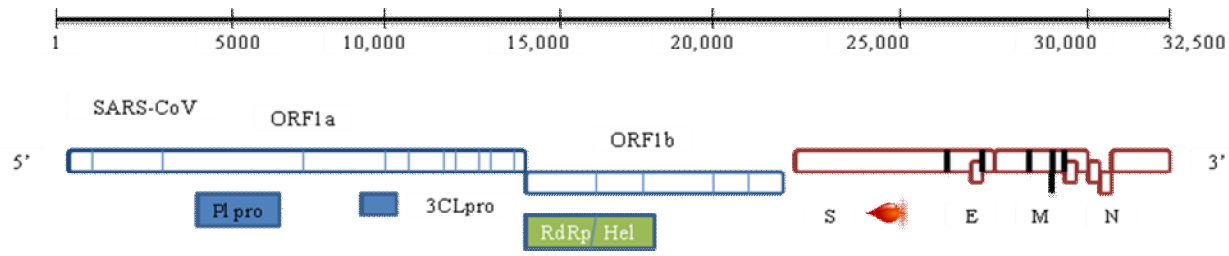
INCEPTION OF CORONA VIRUS

Table 1: Inception of Corona Virus

NAME OF CORONA VIRUS	YEAR
Infectious bronchitis virus (IBV)	**1930**
Mouse Hepatitis Virus (MHV) and Transmissible Gastroenteritis Virus (TGEV)	**1940**
Human Corona Virus BB14	**1960**
Human Corona Virus 229E	**1960**
Human Corona Virus B814 and 229 E	**1967**
Human Corona Virus OC43	**1967**
Human Corona Virus SARS- CoV	**2003**
Human Corona Virus HCoV NL63	**2004**
Human Corona Virus HCoV HKU1	**2005**
Human Corona Virus MERS-CoV	**2012**
Human Corona Virus SARS-CoV- 2	**2019**

CORONAVIRAL STRUCTURE

The structure of coronavirus is generally spherical or pleomorphic, with bulbous projections on the surface. The average diameter of virus particle is approximately 125µm, an envelope is 85µm thick and the spikes are 20µm in length. The genome structure of CoVs is single-stranded RNA. They are usually less than 10kb in length. However, the nCoV genome is much larger (~30kb in length), among the largest known RNA viruses. It has 5' cap structure and a poly-A tail which protects it from nucleases. Typical CoV Genome contains at least 6 Open Reading Frames Genes (ORFs). The first ORF encodes 16 Non- Structural Proteins (NSPs). The envelope of the virus consists of a lipid bilayer having four structural genes as Membranes (M), Envelope (E), Spikes (S) and Nucleocapsid (N) respectively (Figure 1). The ratio of E:S: M in the lipid bilayer is approximately 1:20:300 [40].



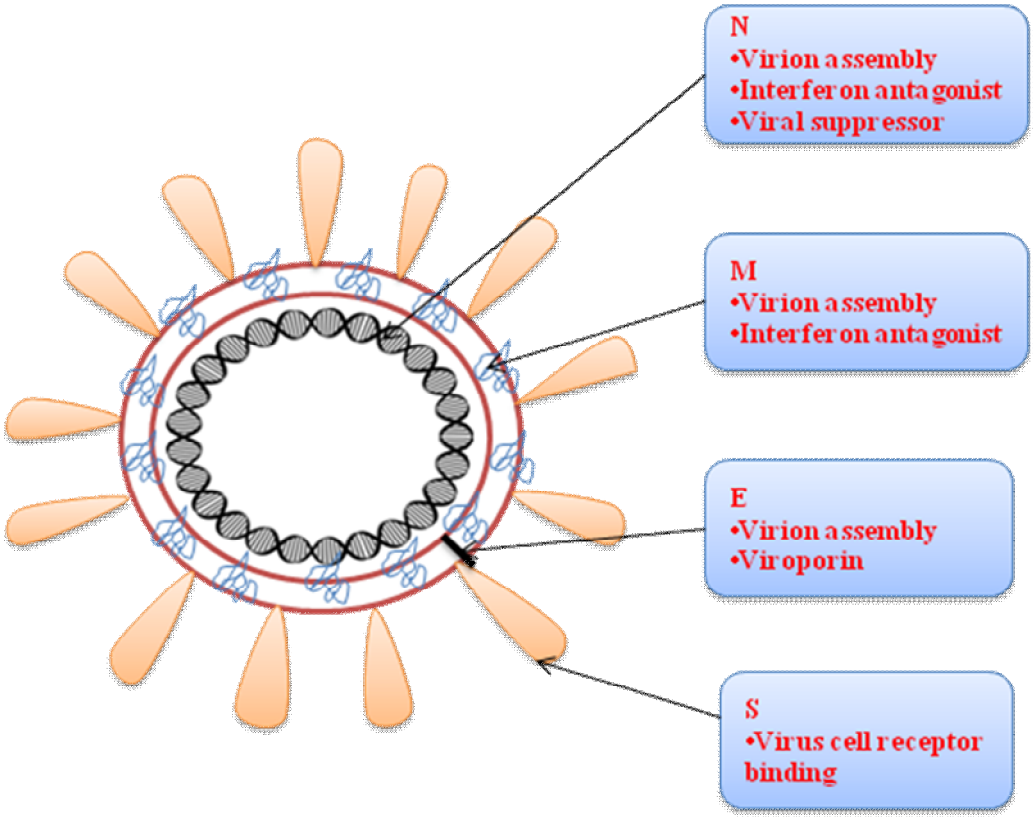


Figure 1: Genomes and structure of SARS-CoV

Spikes (S) - The structure of nCoVs contains 74 spikes present on the surface. The spikes are homotrimers of the S protein, which is composed of S1 and S2 subunit. This is the class I fusion protein which helps in receptor binding and leads to the fusion of membrane between the host cell and the virus. The head of the spike is formed from S1 subunit and also include the Receptor Binding Domain (RBD) whereas the S2 subunit forms the stem which help the spike and activation of protease and thus causes fusion of membrane between the host cell and the virus.

Membranes (M) - It consists of three transmembrane domains. It promotes the curvature of membrane and binds to the nucleocapsid.

Envelope (E) - It plays an important role in the assembly and release of the virus. It is also involved in viral pathogenesis.

Nucleocapsid (N) - Inner part of the virus consist of nucleocapsid, which consists of 2 domains, both of them can bind to the nsp 3 protein which aid in attaching the genome to RTC (Replication- Transcription Complex) and packages the encapsidated genome into virions. A fixed set of subgenomic RNAs (sgRNAs) are synthesised by RTC according to discontinuous transcription. These mRNAs contain 5'-promoter and 3'-terminator sequences [12].

The order Nidovirales are classified among the two sub- family Coronavirinae and Torovirinae [(18)]. However, the coronavirinae is divided into genera- Alpha coronavirus, Beta coronavirus, Delta coronavirus and Gamma coronavirus [38], where Beta coronavirus classified as SARS-CoV, MERS-CoV and SARS-CoV2 [20]. The Coronaviruses, the Toroviruses, the Arteriviridae [39] and the Roniviridae [16, 19], all are grouped under a single order, Nidovirales. They belong to different families from other non-segmented positive-strand RNA viruses as they consist of a higher level of organization. CoVs genome sequence alignment shows approximately 58% identity on the nsp-coding region and 43% identity on the structural protein-coding region in different CoVs, with 54% of a whole-genome level, lead the nsps to be more conserved and the structural proteins are more diverse in need of modification to new hosts. There is a special feature, 3'-5' exoribonuclease which is quirky to CoVs among all RNA viruses as it maintains the function of RTC [12].

TRANSMISSION OF CORONA VIRUS

1. The entry of coronavirus begins when the Spike (S) of a virus which is made up of glycoprotein is attached to its complementary host cell receptor. When attachment takes place, a protease of host cell

cleaves and activates the receptor- attached spike protein. Depending on the availability of host cell protease, the virus enters the host cell through endocytosis or direct fusion of the envelope with the membrane of the host. The RNA genome gets attached to the host cell's ribosome for the process of translation. It translates the beginning of ORF (Open Reading Frame) and forms a long polyprotein which has its protease to cleave polyprotein into multiple non-structural proteins (NSP) [14].

2. Multiple of non-structural proteins assemble to form a multiprotein Replicase- Transcriptase Complex (RTC) with the help of RNA-dependent RNA polymerase (RdRp). It includes replication and transcription of RNA from an RNA strand while other non- structural proteins help in the replication and transcription process except proofreading function which is done by exoribonuclease non-structural protein. The protein N binds to the genomic RNA and the protein M is combined with the membranes of the Endoplasmic reticulum (ER) like the envelope proteins S. After binding, they are enclosed with its membrane. These progenies are transported by Golgi bodies to the cell membranes and are exocytosed into the extracellular space. They are transmitted from one host to another host, through an aerosol or faecal-oral route. This infects human epithelial cells of the respiratory tract (https://www.promega.in) [27].

Clinical Symptoms

Symptoms of Coronavirus disease are classified as Common symptoms, Less common symptoms and Serious symptoms (Table 2).

Table 2: Clinical Symptoms of Coronavirus disease [33].

Category of Symptoms	Symptoms
Common symptoms	• Fever • Dry cough • Tiredness • Pneumonia • Dyspnea
Less common symptoms	• Aches and pains • Sore throat • Diarrhoea • Nausea • Vomiting • Conjunctivitis • Headache • Loss of taste or smell • Skin rashes • Discolouration of fingers and toes
Serious symptoms	• Difficulty in breathing • Chest pain or pressure • Loss of speech or movement • Chills

The incubation period for the infection is of 14 days and the age group affected includes Adults of median age (49 to 56 years), older age with age related ailments (Cardiovascular, Renal disease, Hypertension, Chronic lung disease, Cancer etc.).

Materials and Methodology

Beginning from the articles found in the recent searches related to this, a snowball strategy of search is used in which references and articles of relevance are used for the study. Moreover, experts were conferred for some more articles related to this. This search was last updated in the last week of April.

With rapidly increasing cases of Covid-19, the diagnostic test to penalize this has been developed to cure this epidemic disease.

There are five diagnostic procedures for SARS CoV-2.

1. Molecular Assay (RT-PCR Test) - A Direct Method
2. Serological Test (Antibody Test) - An Indirect Method

3. Antigen Detection test
4. Isothermal amplification assays and
5. Medical imaging

ASPECT OF DIAGNOSTIC TESTING IN THE COVID-19 EPIDEMIC

The first stage of testing is sample collection.

Sample type depends on the test being conducted.

A) **Naso-pharyngeal or oropharyngeal swab for an RT-PCR test**: In the initial week of infection, coronavirus mainly replicates in the upper parts of the respiratory system (nose, mouth, and throat). For sampling, a healthcare technician uses a **special nylon/dacron swab** to collect the sample from the back of the throat and/or nose. Swabs containing Calcium Alginate, Wood, or cotton should be avoided because they may contain certain substances that inhibit PCR testing. The swab is put into a tube having a liquid (called the **viral transport medium, VTM or Universal Transport medium, UTM**) (mainly Sucrose based). Inadequate sample collection may result in a false-negative test. The sample is packed well into 3 layers of protection and sent to the investigating laboratory (https://www.promega.in) [27].

B) **Blood sample** for an antibody test: The two main antibodies, IgG and IgM, that form the basis of most antibody tests, usually circulate in the blood to be tested.

Different diagnostic kits from various companies identify different genes of SARS-CoV-2 such as E gene, RdRp gene, N gene, ORF and S gene (Table 3).

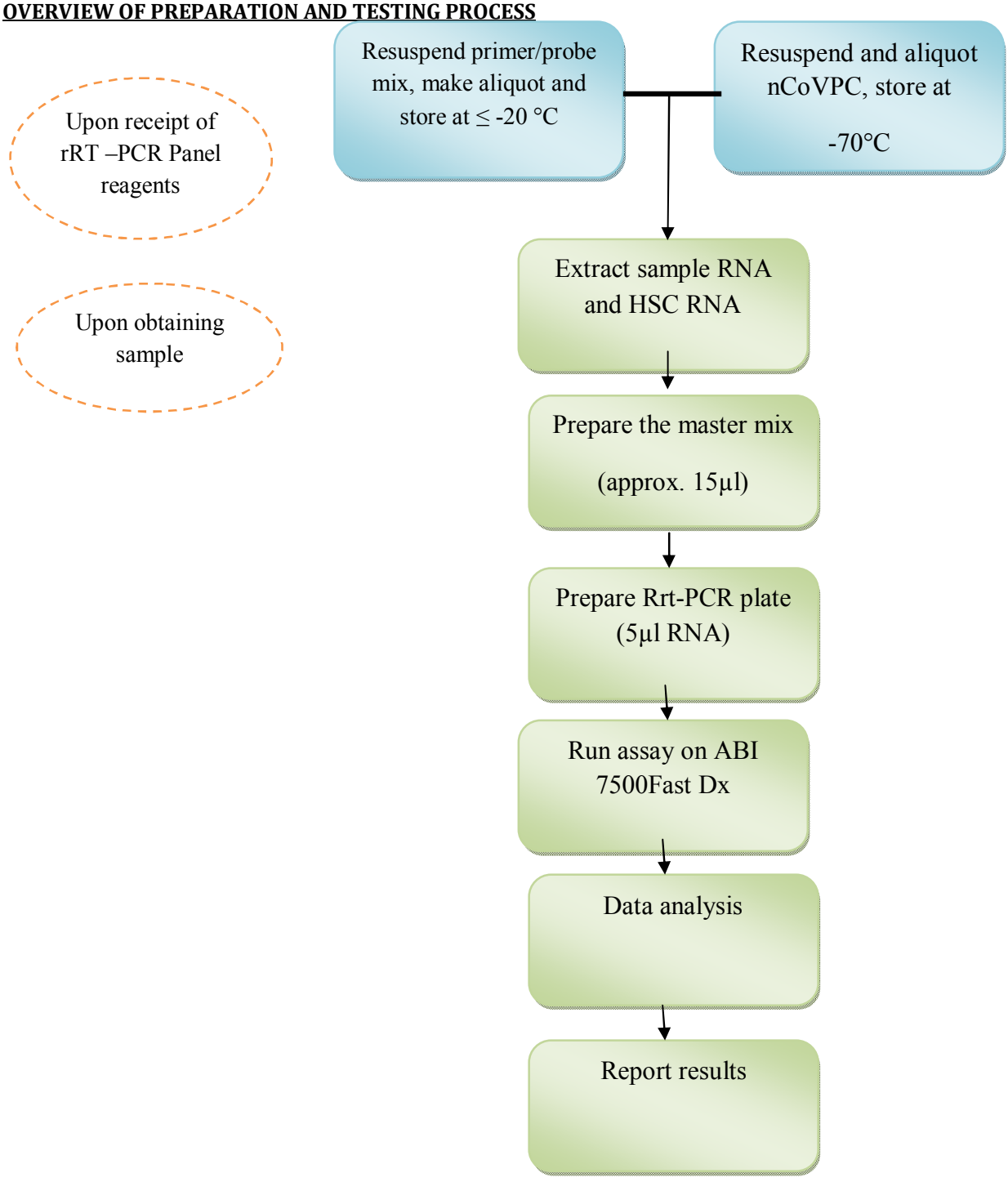
Table: 3 Kits from various companies identifies different genes of SARS-CoV-2

COVID-19 Gene Targets	NIV Pune	TRUPCR 3B	Thermo	Qiagen	Altona	Roche	MyLab
ORF 1ab			Yes			Yes	
N		Yes	Yes				
ORF 1b-nsp 14	Yes			Yes			
RdRp	Yes	Yes					Yes
E	Yes	Yes		Yes	Yes	Yes	Yes
S			Yes		Yes		

Molecular Assay (RT-PCR Test) - A Direct Method- Molecular assay (RT-PCR test) is a laboratory examination involving reverse transcription of mRNA into cDNA and amplify target DNA through Polymerase Chain Reaction (PCR). It is used to measure the amount of specific RNA. This is done by observing the amplification reaction through fluorescence, a technique called Real-Time PCR/ Quantitative PCR (qPCR). The probe contains a fluorescent dye molecule on 5'end and a quencher molecule on its 3' end [21].. This method includes the following steps namely viz-

- Extraction of RNA (Genetic Material)
- Reverse Transcription and Amplification
- Interpretation of results

OVERVIEW OF PREPARATION AND TESTING PROCESS



The genetic material of Covid-19 i.e. single-stranded RNA is extracted either manually or automated with the help of RNA extraction kits developed by various manufactures (Table 4). The extracted viral RNA is then converted to cDNA by the enzyme Reverse Transcriptase. This ssDNA (cDNA) gets converted into dsDNA with the help of DNA polymerase. Primers (Forward and Reverse), Probe (Fluorofore and Quencher molecules), dNTPs and Taq DNA Polymerase further amplifies the dsDNA into multiple copies (40 cycles). During this process, Taq DNA Polymerase has 5'-3' exonuclease activity, by which it cleaves the probe molecule (TRUPCR SARS- CoV- 2 RT qPCR Kit. www.3bblackbio.com., 2020) [42]. Several manufactures develop their RT-PCR diagnosis kits with different protocols.

Table 4: Instruments and Extraction kits

INSTRUMENT	EXTRACTION KIT
QIAGEN	QIAmp DSP Viral RNA Mini Kit
	QIAmp Viral RNA Mini Kit
QIAGEN EZ1 Advanced XL	EZ1 DSP Virus Kit
	EZ1 Virus Mini Kit v2.0
Roche MagNA Pure LC	**Roche MagNA Pure LC DNA Isolation Kit**
Roche MagNA Pure Compact	**Magna Pure Compact Nucleic Acid Isolation Kit**
Roche Magna Pure 96	**Magna Pure 96 Cellular RNA Kit**
QIAGEN QIACUBE	

According to **CDC (Centres for Disease Control and Prevention),** Molecular assay (rRT-PCR) is a test utilized for the detection of quality of nucleic acid from the 2019- nCoV in swabs collected from the upper and lower respiratory tract of the individuals showing 2019-nCoV clinical and/or epidemiological criteria (for example, clinical signs and symptoms associated with 2019-nCoV cases, or other epidemiologic links for which 2019- nCoV testing may be indicated as a part of a public health investigation). Active infection of 2019-nCoV shows positive results but do not exclude the bacterial or other viral infections negative results are correlated with clinical symptoms as well as contact history (CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel) [8].

SET UP ASSAY

Protocols for the molecular assay of the nCoV RNA are designed by several manufacturers (**Thermofisher, Quantabio etc)** of the diagnostic kits according to their primers and probes (Table 5,6,7,8).

Table 5: ThermofisherTaqPath 1-step RT-qPCR Master Mix

STEP	REAGENT	VOLUME OF REAGENT ADDED PER REACTION
1	Nuclease-free water	N×8.5 µl
2	Combined primer/ probe mix	N×1.5 µl
3	TaqPath 1-step RT-qPCR Master Mix (4x)	N×5.0 µl
	Total volume	N×15.0µl

Table 6: Promega Go Taq Probe 1-Step RT-qPCR System

STEP	REAGENT	VOLUME OF REAGENT ADDED PER REACTION
1	Nuclease-free water	N×3.1 µl
2	Combined primer/probe Mix	N×1.5 µl
3	Go Taq Probe qPCR Master Mix with dUTP	N×10.0 µl
4	Go Script RT Mix for 1 step RT- qPCR	N×0.4µl
	Total volume	N×15.0 µl

Table 7: Quantabioq Script XLT one-step RT-qPCR ToughMix

STEP	REAGENT	VOLUME OF REAGENT ADDED PER REACTION
1	Nuclease- free water	N×3.5 µl
2	Combined primer/probe Mix	N×1.5 µL
3	qScript XLT one-step RT-qPCR Tough Mix (2x)	N×10.0 µl
	Total volume	N×15.0 µl

Table 8 : QuantabioUltraPlex 1-step ToughMix (4x)

STEP	REAGENT	VOLUME OF REAGENT ADDED PER REACTION
1	Nuclease-free water	N×8.5 µl
2	Combined primer/probe Mix	N×1.5 µl
3	UltraPlex 1-step ToughMix (4x)	N×5.0 µl
	Total volume	N×15.0 µl

INTERPRETATION OF RESULTS

In a negative control reaction, already set probe/primer does not exceed the threshold line of the fluorescent growth curve. If one or more primers and probe non-template control (NTC) reactions occur, then the sample is contaminated and a false positive case occurs. Ct values for positive control reaction varies (22±5) according to the probe/ primer of the manufacturer's protocol (Table-9). A sample with 35 Ct value shall be considered as a positive. Primarily, the fluorophore dye is cleaved from 5' end of the probe which gives fluorescence and finally quencher molecule is separated which quenches fluorophore. The total fluorescence of reaction volume increases in direct proportion to the number of amplicon copies synthesized during PCR [1, 7]. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The fluorescent signal is measured in each cycle of reaction and the threshold cycle value is determined from the obtained curve. The threshold cycle is proportional to the initial number of RNA copies in a sample (Figure 2). Amplification of E gene detects Sarbeco virus and structural genes like RdRp gene, N gene, ORF 1 ab, ORF 1b-nsp 14 and S gene confirm the presence of SARS-CoV-2 (TRUPCR SARS- CoV- 2 RT qPCR Kit. [42].

Table 9: Expected performance of controls

CONTROL TYPE	EXTERNAL CONTROL NAME	USED TO MONITOR	2019 nCoV_N1	2019 NCoV_N2	RP	Expected Ct value
Positive	nCoVPC	Substantial reagent failure including primer and probe integrity	+	+	+	<40.00 Cr
Negative	NTC	Reagent and environmental contamination	-	-	-	None detected
Extraction	HSC	Failure in lysis and extraction procedure, potential contamination during extraction.	-	-	+	<40.00Cr

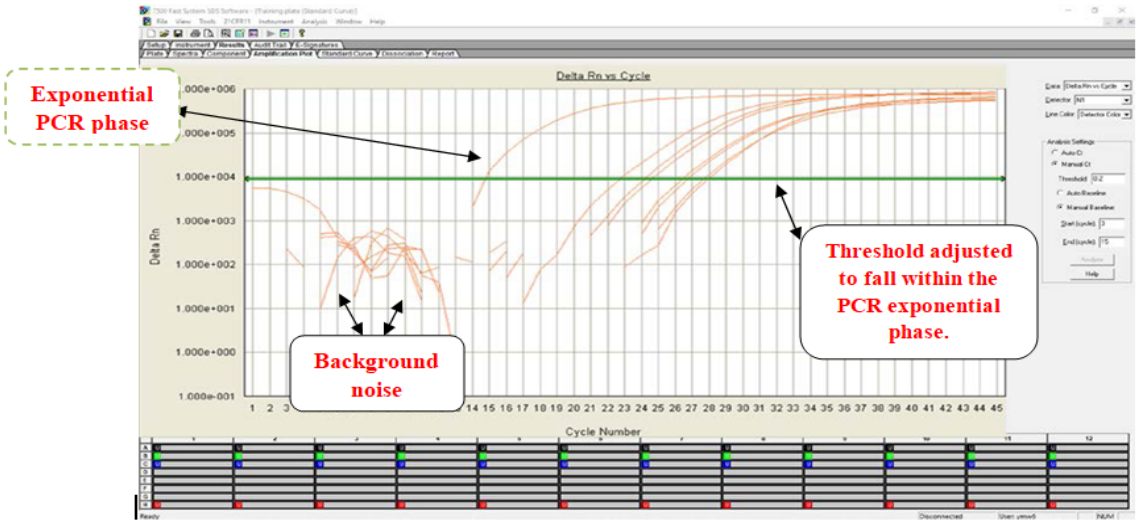


Figure 2: Interpretation of Results

DETECTION OF RSV AND INFLUENZA B RNA

For the detection of viral RNA UTM is immunized with the nasopharyngeal swab and spiked with RSV A and Influenza B virus. Virus sample with high UTM contains approximately 2×10^5 copies of RSV A and Influenza B both per sample whereas low UTM sample is a 1:100 dilution of the high virus sample.

Serology Test (Antibody Test)

Antibody tests may be used for testing suspected people in the identified hot spot areas and containment zones. Lymphocytes in our body secrete Antibodies (γ-globulin) which is the first line of protection against any infection. IgM (Largest Ig) is the first antibody to be made by the body (acquired immunity) to fight against a new infection (Figure 3) [23].

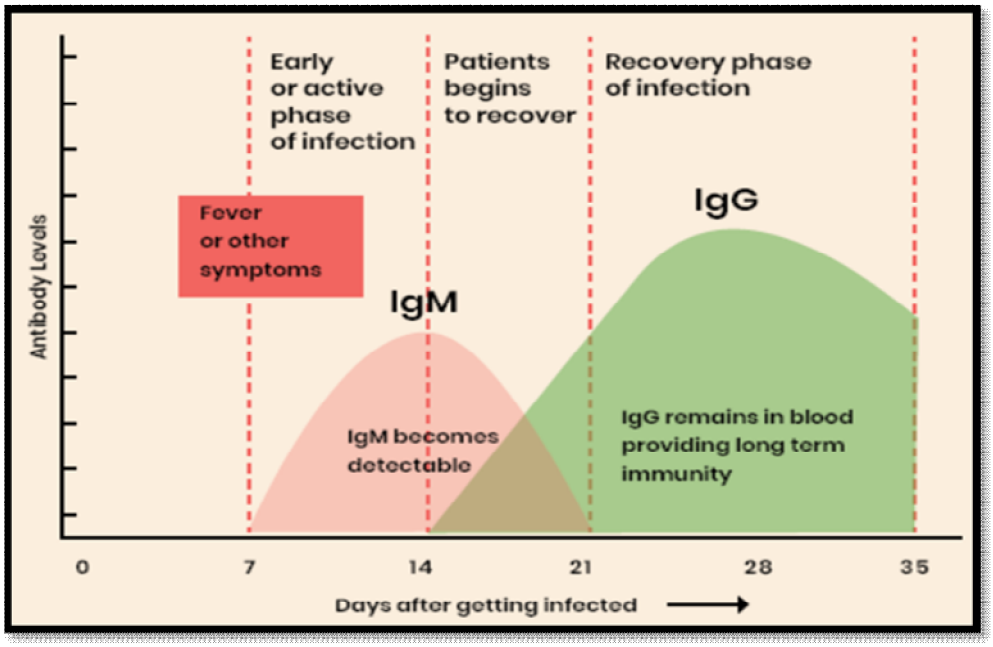


Figure 3: Antibodies produced by the body during coronavirus infection.

IgG is an antibody with a good memory. It provides long-term immunity. It remembers which germ has infected the individual in the past. When the coronavirus enters human body, the IgM level will rise for a short time. It will then begin to drop as the IgG level increases and helps to fight against (secondary response) the infection [32]. Correlation and difference between molecular assay and antibody test are depicted in **Figure 4** [15, 23]. After recovery from Covid-19 infection molecular assay give a negative result while antibody test results in a positive outcome due to the presence of IgG in the blood.

Parameters	rRT-PCR	Antibody kits
Type of sample	Nose and/or throat swab (most common)	Blood/ serum (fluid component of blood)/ swab
Detection Type	Genes of novel coronavirus	Antibodies in body
Sample processing time	Around 6 hours	Less than 30 minutes**
Time when the test can yield results	1-3 days after getting infected	5-10 days after getting infected
Usage	• Used for confirmation of COVID-19 • Highly sensitive	• Used for screening in hot spot areas and also understand the immune status of population • Less sensitive than rRT-PCR

Figure 4: Difference between rRT- PCR and Antibody kits

Antigen Detection test

This immunoassay directly shows low complexity and may provide results earlier at the point of care. In the present scenario, the sensitivity of this test is suboptimal to rule out the disease. Samples of such tests for other novel coronaviruses are still under process while monoclonal antibodies against the nucleocapsid protein of SARS-CoV-2 have been generated, which lead to the upcoming rapid antigen detection test [14].

Isothermal amplification assays

Uses an **isothermal gene amplification method,** which does not need alternating temperatures, hence saves time and cheapest one. If the sample is positive, gives results in just 5 minutes. If the sample is negative, gives results within 15 minutes.

It is a single-tube technique for amplification of a gene at a constant temperature of 60-65°C by using either two or three primer sets and a polymerase with high strand displacement activity. In this, 4 different types of primers are used to amplify 6 different regions on the target gene which increases the specificity and ruggedness. The amplification product can be detected which depends upon photometry and turbidity measurement (magnesium pyrophosphate precipitate in solution) or by fluorescence using intercalating dyes such as SYTO 9 [35].

Medical imaging

Chest imaging may also be used for diagnosis. In chest radiography, the most important one is bilateral pneumonia. Computed tomography is regarded as most sensitive than radiography.

GeneXpert

Close to 500 existing instruments, called GeneXpert, which are in use to detect tuberculosis, are now being re-purposed for COVID-19 testing. This will immediately increase the testing ability in the country. It is a cartridge-based technique. To start the test, VTM liquid containing the sample is pipetted into a disposable test cartridge, and the cartridge is inserted into the test machine; this takes no special training. After this, the process is automatic, and hence quicker**,** and provides the result within 45 minutes [30, 26].

VACCINE DEVELOPMENT

Multiple vaccines are being assessed for the prevention of COVID-19 [46]. The first vaccine to be studied in humans in the United States used the mRNA for the expression of the viral spike protein to indure an immune response [24]. A Bacille-Calmette-Guerin (BCG) immunization is generally used for prevention of COVID-19. WHO updated its review on 11 April 2020 related to BCG vaccination in which authors compared the different countries with BCG vaccine and its usage [45]. Its primary motive is to penalize tuberculosis, BCG immunization persuades a non-specific immune response that defends the effects against non- mycobacterial, including viral, infections[2,34]. According to WHO, BCG vaccination cannot be used for the prevention of COVID-19, as there is no evidence related to this vaccine and suggested continued use of this vaccine in countries with high number of tuberculosis cases [36, 5, 9, 17, 37].

PREVENTION

To prevent infection of COVID-19, do the following:

- Wash your hands regularly with soap, or clean them with alcohol-based hand rub or sanitizer.
- Maintain at least 1-metre distance between you and the surrounding peoples.
- Cover your mouth and nose when coughing or sneezing through handkerchief or elbow.
- Avoid touching your face and nose frequently.
- Avoid unnecessary travelling and staying away from large groups of peoples.
- Abstain from smoking and other activities (https://www.who.int).

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