# We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

**5,300**
Open access books available

**130,000**
International authors and editors

**155M**
Downloads

Our authors are among the

**154**
Countries delivered to

**TOP 1%**
most cited scientists

**12.2%**
Contributors from top 500 universities

CLARIVATE ANALYTICS BOOK CITATION INDEX INDEXED

WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index in Web of Science™ Core Collection (BKCI)

## Interested in publishing with us?
## Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com

Chapter

# Development of RT-PCR Based Diagnosis of SARS-CoV-2

*Rutuja Sunil Patankar and Vasudeo Pandharinath Zambare*

## Abstract

In the 2020, COVID-19 pandemic disease created an havoc situation world widely and mainly caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). It has been challenging task for researchers, scientists and medico-pharmaceutical organisations to find out rapid and reliable diagnosis methods. Among the all testing services, a Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the more accurate, rapid and authenticated molecular technique used for most of the diagnosis of major diseases. It has been a global priority to fix the rapid diagnosis method to combat against the pandemic COVID-19. Thus, the present chapter mainly focussing on the progress of RT-PCR method development though various processes of data collection on isolation of whole genome sequence, its primer and method designing. In this scenario, India suddenly become the global leader for vaccine development and hence the challenges and RT-PCR kit development in India and rest of the world has been be discussed. World wide many Government and private agencies and industries have taken an initiative for diagnosis of SARS-CoV-2 hence this chapter also summarised the scope of RT-PCR to combat pandemic situation in future.

**Keywords:** diagnosis, RT-PCR, COVID-19, SARS-CoV-2, Primers

## 1. Introduction

Coronavirus outbreak first case found in Wuhan, China in December 2019 [1]. Further, it became a pandemic, affecting the whole world. On February 2020, World Health Organisation (WHO) announced an official name for Coronavirus spread disease as COVID-19 [2]. It primarily targets a respiratory system in humans, as the appearance of symptoms depends on the incubation period, which further relies on the patient's age and the immune system [3].

SARS-CoV-2 as a public health emergency was declared by WHO and thereafter it became essential to find diagnostic tests for early detection and early treatment [4]. Normally the best way to detect any virus from the sample is its isolation and further confirmation by various molecular techniques. But Center for Diseases Control and Prevention (CDC) recommended not to isolate the SARS-CoV-2 as it is a new virus and this practices could be a risky approach and suggested to use patients sample directly for diagnosis [5]. Most of the diagnostic methods are molecular-based hence for diagnosis virus genome study become necessary. On January 2020 the ssRNA - 29870 bp whole sequence of SARS-CoV-2 was reported with GenBank accession number MN908947 [6, 7]. Thus, the basic information

of genome sequence helpful for molecular based detection method development specially Polymerase chain reaction (PCR) based methods.

Main focused was on PCR as it is a rapid detection method. Among that Reverse Transcription Polymerase Chain Reaction (RT-PCR) is the most commonly used and still be using the method as it is exactly showing the positive results with great -sensitivity. RT-PCR was said to be a gold-standard for testing coronavirus [8]. RT-PCR can detect the virus from throat, nasopharyngeal swab as well as from stool sample [9]. Early detection using PCR prove to be most sensitive way as negative sample cannot be ruled out. One cohort study found that patients even if not having any symptoms still may be the carrier for viruses thus RT-PCR can detect those at genetic level. Thus, it helps in prevention of nosocomial infection and further spread unknowingly [10].

Sample collection methods also affect the diagnosis results. Thus, WHO and CDC recommended the use of nylon, rayon or any synthetic fibre swabs for sample collection. But shortage of such swabs hammered the testing numbers. Hence, some researchers decided to study the effect of cotton tipped plastic swabs on PCR results and it found that no inhibition effect was seen on PCR [11]. Another method related to heating of sample known as Loop-mediated Isothermal Amplification (LAMP) this works by skipping the RNA extraction process. RNA RT-LAMP sensitivity found to be 97%, while specificity was 99% [12]. Study tried the three viral transmission medium heating treatments named as directly without additives, in a formamide-EDTA buffer and in a RNAsnap™ buffer. Basically, this method skips the RNA extraction process of RT-qPCR by replacing it with heating treatments thus reduces the time for the test and also gives the same result as normal RT-PCR protocol [13]. Similarly, in one study RNA extraction process was skipped and on that place RT-qPCR master mixes were used. Now this helps under such situation where reagent shortage occurs in hospitals. It also expanded the testing capacities [14]. Similar way direct RT-qPCR was used in one of the study found to be an alternative for classical method without RNA extraction [15]. But there are certain limitations in remote areas and portable diagnosis will be preferable using mini PCR based diagnosis kits. One study combined the mini PCR and multi-well plate reader for convenient and portable diagnosis under pandemic situation [16].

As per the available RNA sequences and need of diagnosis method for COVID-19, several RNA extraction kits are available in the market. But due to pandemic situation and rising number of COVID patients, it was under shortage. Thus, magnetic beads base RNA extraction methods were used by various researchers. Silica beads were found to yields RNA and comparable with commercially available QIAcube viral RNA extraction kit which were determined by RT-qPCR and RT-LAMP [17]. Another study due to complexity of protocol was done, in which rather than using transport medium and RNA extraction it was decided to study the use of direct elution of swab and performing RT-qPCR. Elution of dry swabs were done directly in simple TE buffer and tested. Thus, this process simplifies the pre RT-qPCR preparation [18].

Commercial RT-PCR kits are already available in the market and few of them are manufactured by Altona Diagnostics (Hamburg, Germany), BGI (Shenzhen, China), CerTest Biotec (Zaragoza, Spain), KH Medical (Gyeonggi-do, Republic of Korea), Primer Design (Chandler's Ford, UK), R-Biopharm AG (Darmstadt, Germany), and Seegene (Seoul, South Korea). All these kits are the best for detection without any cross-reactivity with another virus and hence can be used for regular diagnosis of SARS-CoV-2 [19]. But the limit of detection (LoD) of kits matters the quality. Earlier China National Medical Products Administration (NMPA) approved 6 PCR kits, but due to time shortage optimisation was not done so LoD might get affected. Thus

examination of LoD of this 6 PCR kits with real RNA of the virus was carried. All kits showed different LoD and the poorest LoDs has high chances of giving false negative results. The lab should confirm the performance of the kits and the utilise [20]. Some of the primers and probes for CoV detection has been shown in **Table 1**.

Currently, the RT-PCR test has a 95% specificity and 70% of the sensitivity rate [28, 29]. Self-collected saliva specimens was tested with 6 different molecular diagnostic tests like RTqPCR LDT, SARS-CoV-2 RAT, 3 direct RT-qPCR kits, and RT-LAMP and all tests showed a excellent results. Thus, based on these results, molecular diagnostic method has a great scope ahead [30]. Dr. Aneesh Mehta said that saliva test for coronavirus by PCR is the new type of diagnosis, as it is not invasive method but just spit is required as a sample [31]. This test is said to be a 'SalivaDirect' test developed by a scientist of the Yale School of Public Health, authorised by Food and Drug Administration (FDA) [32].

Most countries have been used Indian Council of Medical Research (ICMR, India) suggested Rapid Antigen Test (RAT) which was available as a kit and direct antigen identification from nasal swab [33]. However several countries found that the negative findings by RAT were shown to be positive by RT-PCR. Thus, according to Dr. Balram Bhargava, Director of ICMR, the RAT was found to offer false-negative outcomes. One of the reports says that around 11% of people found negative by RAT in Delhi, after testing with RT-PCR found to be positive [34]. The Indian Health Ministry and ICMR provided a guideline to re-test the RAT tested negative patients and those who develop symptoms after a few days of the test by RT-PCR [35]. Until now, RT-PCR is the recommended test for all organisations and has been followed. Still, many experts are trying to carry out a number of inventions and studies to make it simpler, more practical and more cost-effective for the whole planet. This book chapter summarised the upto date basics and applied study

| Country | Target genes | Sequence | Reference |
|---|---|---|---|
| China | ORF1a | Forward primer: AGAAGATTGGTTAGATGATGATAGT<br>Reverse primer: TTCCATCTCTAATTGAGGTTGAACC<br>Probe:FAM-TCCTCACTGCCGTCTTGTTGACCA-BHQ1 | [21] |
| Hong Kong | N gene | Forward primer: TAATCAGACAAGGAACTGATTA<br>Reverse primer: CGAAGGTGTGACTTCCATG<br>Probe: FAM/ZEN-GCAAATTGTGCAATTTGCGG-IBFQ | [22] |
| USA | N1 gene | Forward primer: GAC CCC AAA ATCAGCGAA AT<br>Reverse primer: TCTGGTTACTGCCAGTTGAATCTG<br>Probe: FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1 | [23] |
| USA | N2 gene | Forward primer: TTACAA ACATTGGCCGCA AA<br>Reverse primer: GCGCGACATTCCGAAGAA<br>Probe: FAM-ACA ATTTGCCCCCAGCGTTAG-BHQ1 | [24] |
| USA | N3 gene | Forward primer: GGGAGCCTTGAA TAC ACC AAA A<br>Reverse primer: TGTAGCACG ATTGCAGCATTG<br>Probe: FAM-AYCACATTGGCACCCGCA ATCCTG-BHQ1 | [25] |
| Germany | E gene | Forward primer: ACAGGTACGTTAATAGTTAATAGCGT<br>Reverse primer: ATATTGCAGCAGTACGCACACA<br>Probe: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ | [26] |
| China | Spike | Forward primer:<br>CCTACTAAATTAAATGATCTCTGCTTTACT<br>Reverse primer: CAAGCTATAACGCAGCCTGTA | [27] |

**Table 1.**
*Primers and probes recorded for SARS-CoV-2 real-time reverse transcription PCR assays.*

of PCR based diagnosis of SARs CoV-2 including current challenges of diagnosis, protocols and future prospects.

## 2. Challenges for SARs CoV-2 diagnosis

For treatment of SARs CoV-2, no vaccines is available but it was only the option left out to healthcare sectors that to prevent transmission as soon as possible [26]. So most the countries invested in isolation and detection [26]. The diagnosis of COVID-19 was difficult as it shows symptoms similar to those of flu viruses, thus it was crucial to find the diagnosis as early as possible for management purpose [36]. One of the most important problems in analytical chemistry was highlighted by the COVID-19 pandemic outbreak: the discrepancies between the testing technique (TT) and the testing method (TM) are a common confusion in the clinical field. In addition to the research procedure, TT consists of many steps, such as the collection of specimens, their preservation, storage, transport, labelling and distribution. Pre-test planning procedures for patients are also part of the process. Previously, these procedures, also known as pre-analytical variables, have been identified as the key causes of laboratory testing errors. The most acceptable TT for the TM must be validated during the production of the TM for the identification of the target analyte; otherwise the analysis is performed within a wide range of analytical errors [37]. Successful detection of the virus also depends on time of testing, early or late detection, viral load, sample collection etc. [38].

Further challenge was during the false negative results. It is easy to understand and interpret a perfect test for a disease; the test would only be positive if the disease was present, and it would only be negative if it were absent. However, since all studies have false positives and false negative results, diagnostic tests are not flawless. Test results do not definitively state whether or not there is a disease (or virus). This does not mean that the test is not beneficial; it merely implies that the test results must be probabilistically tested on the basis of test output characteristics, patient data, and disease prevalence [39]. To interpret the results of incomplete tests, two main metrics that characterise the test are needed: diagnostic sensitivity and diagnosis specificity [40]. At present, for commonly used SARS-CoV-2 samples, there is minimal information on these values. To accurately interpret an incomplete test, the approximate probability that the person being evaluated has the disorder must also be considered [39].

The diagnosis was completely relied on two ways Molecular and serological testing as shown in **Figure 1**. Different test among these two categories was tried during a pandemic situation [41].

Currently, RT-PCR is commonly used method by many laboratories due to specificity and fast detection [40]. Some test commonly use for CoV detection has been shown in **Table 2.**

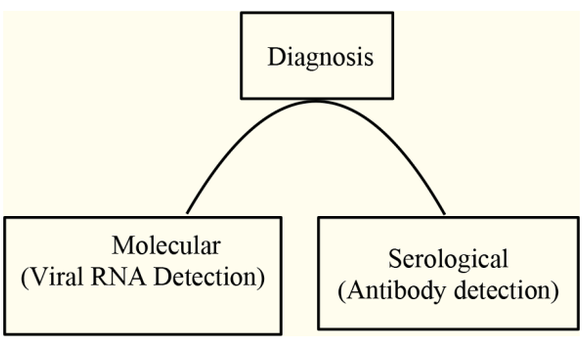


**Figure 1.**
*Two ways of COVID-19 diagnosis.*

| Tests | Detection | Sample | Advantage | Disadvantage | Reference |
|---|---|---|---|---|---|
| **Molecular** | | | | | |
| RT-PCR | Viral RNA | Nose, throat swab, sputum, stool | Specific Less time | False negative result if viral load is less or if sample is not collected properly. | [24] |
| Nested RT-PCR | Viral RNA | Nose, throat swab | Highly specific results even in low viral load sample | Cross contamination during protocol | [41] |
| RT-LAMP | Viral RNA | Nose, throat swab, sputum, stool | Specific Less time | Optimize reaction condition | [42] |
| CRISPR | Viral RNA | Nose, throat swab, Broncho alveolar lavage fluid | Easy to use Low cost | Can give false result | [8] |
| Viral sequencing | Viral RNA | Nose, throat swab | Sensitive, convenient | Costly, need trained person, sophisticated instrument | [43] |
| **Serology** | | | | | |
| ELISA | Antibody | Blood | Simple and cost effective | Not established for SARS-CoV-2 | [44] |
| Neutralization assay | Antibody | Whole blood, serum, plasma | Quantitative result, | Live virus hence safety required | [37] |

**Table 2.**
*Diagnostic testing for SARS-CoV-2.*

## 3. Real-time RT-PCR basics

RT-PCR stands for real-time reverse transcription-polymerase chain reaction (RT-PCR). It is a technique use to determine the nucleic acid (DNA/RNA) from sample specifically from a virus or bacteria. Currently used for COVID-19 testing. It combines the reverse transcription of RNA into DNA It uses radioactive isotope marker or fluorescent dyes for detection of the targeted gene. It can detect genetic material from nose, throat, stool and sputum sample [45].

## 4. Real-time RT-PCR protocol

SARS-CoV-2 testing solely depends on patients' health and concern, if the patient is getting some symptoms like cough, fever, headache or related, then the patient can personally take a test by visiting COVID center. If patient is already under medical treatment related to some other health issues, then doctor can recommend a COVID test to that patient for an early treatment. After that the most important part of RT-PCR covid testing is a collection of sample. It is most

important as if the sample is not collected properly, it can affect the result as seen in many research [46–48]. CDC has provided the protocol for sample collection and all labs are following this same. Along with that, instruction regarding virus isolation has been given, it is not suppose to be done by an unless it is performed in the BSL-3 laboratory. Sample recommended for collection are nasopharyngeal (NP) and oropharyngeal (OP) swab for RT-PCR [49]. An expert technician can collect the sample from the nose and throat of patient separately by using swab which is individually wrapped. Swab has to be made up of synthetic fiber like plastic or wire shafts. Technician has to follow certain rules while collecting sample like 6 feet of separation, personal protective equipment (PPE) kit, proper gloves and faced covered lid. The sample is to be collected deeply. Once collected it has to be transferred to viral transport medium (VTM). VTM is made up of 2% FBS, 100 μg/mL Gentamicin and 0.5 μg/mL Amphotericin B. Among this 3 ml of media is transferred to sterile screwed capped bottles in which swab is put and stored before further testing [50]. Sample can be stored at 2–8 °C for 72 hours only. If the sample has to be transported for a long distance, then it should be carried in icepack [51].

Once the sample has been collected, then next important and time consuming process is RNA extraction means purification of RNA samples. Most of RNA extraction kits are available in the market shown in **Table 3**, which can be used directly contain lysis buffer and other chemicals which will lyse the virus and RNA will get into solution. First, in order to release the genetic material, the patient's sample is

| Kits | Company | Country |
|---|---|---|
| **Virus RNA Extraction kit** | | |
| QIAamp DSP Viral RNA Mini Kit | Qiagen | Hong Kong, Japan, USA |
| QIAamp Viral RNA Mini Kit | | |
| Chemagic Viral DNA/RNA 300 Kit H96 | PerkinElmer chemagen Technologie GmbH | Germany |
| SARS-CoV-2 Nucleic Acid Kit (RUO) | | |
| MagNA Pure 96 System | Roche | Germany |
| ANDiS Viral Nucleic RNA Auto Extraction & Purification Kit | 3Dmed | China |
| NucleoMag Dx Pathogen Kit | Macherey-Nagel | France, USA, Switzerland |
| NucleoSpin RNA Virus | | |
| NucleoMag Virus kit | | |
| **Detection kit** | | |
| SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase | ThermoFisher | USA |
| TaqMan Fast Virus 1-Step Master Mix | | |
| SuperScript III Platinum One-Step qRT-PCR Kit | | |
| TaqPath 1-Step RT-qPCR Master Mix, CG | | |
| QuantiTect Probe RT-PCR Kit | Qiagen | Japan |
| New Coronavirus Nucleic Acid Detection Kit | PerkinElmer chemagen Technologie GmbH | Germany |
| Respiratory SARS-CoV-2 RT-PCR Panel Assay | | |
| SARS-CoV-2 Real-time RT-PCR Assay | | |
| EURORealTime™ SARS-CoV-2 | | |

**Table 3.**
*Virus extraction and detection kits.*

mixed with a solution which lyses the cells. In order to purify RNA, inactivation of RNA activity, denaturation of nucleoprotein complexes and elimination of contaminating DNA and proteins must take place by (phenol which will attract the other protein and break it down, guanidine isothyocynate is also a protein denaturant, RNase inhibitors to inactivate the ribonuclease enzyme) [52]. The resulting cellular debris will then proceed to the RT-PCR stage with the extracted RNA. Further RNA purification is carried by using kit or by centrifugation and solid phase extraction by using column Centrifuge and spin column, which is to be placed in a clean collection tube to collect the supernatant and filtrate is discarded. Again, it has to be washed and centrifuge. Further by using elution buffer RNA is purified.

After purification of viral RNA, the next step is the preparation of the reaction mixture for PCR amplification. In this step master mix has to be used which is premixed concentrated solution that consists of buffer, Reverse transcriptase enzyme nucleotide, forward primer, reverse primer, TaqMan probe, DNA polymerase. Finally, the RNA template to be added and mixed by pulse vortexing. Then load the reaction mixture into a PCR plate which generally contain 96 wells, allowing analysis of several samples at a time. Then place this plate in PCR machine (thermal cycler). Real time RT-PCR is used for detection of new Coronavirus 2019 by amplification of target sequences in Rdrp genes, E gene and N gene. The choice of the target depends on primers and the probe sequences. The first step in RT-PCR is reverse transcription. The first strand complementary DNA synthesis is primed with the PCR reverse primer which hybridizes the complementary part of the virus RNA genome. Reverse transcriptase then add DNA nucleotides onto the 3-prime end of the primer synthesizing DNA complementary of the viral RNA. Then denaturation takes place. Thus PCR consists of a series of cycles consisting of Denaturation, annealing and elongation. In cycle 1, DNA denaturation at 95 °C occurs. The next step at 58 °C allows the annealing of forward primer to complementary part of DNA. In elongation step, DNA polymerase synthesize a new strand complementary to the DNA template by adding nucleotides from the reaction mixture. In 2nd cycle, DNA denaturation form ssDNA, then annealing of primers

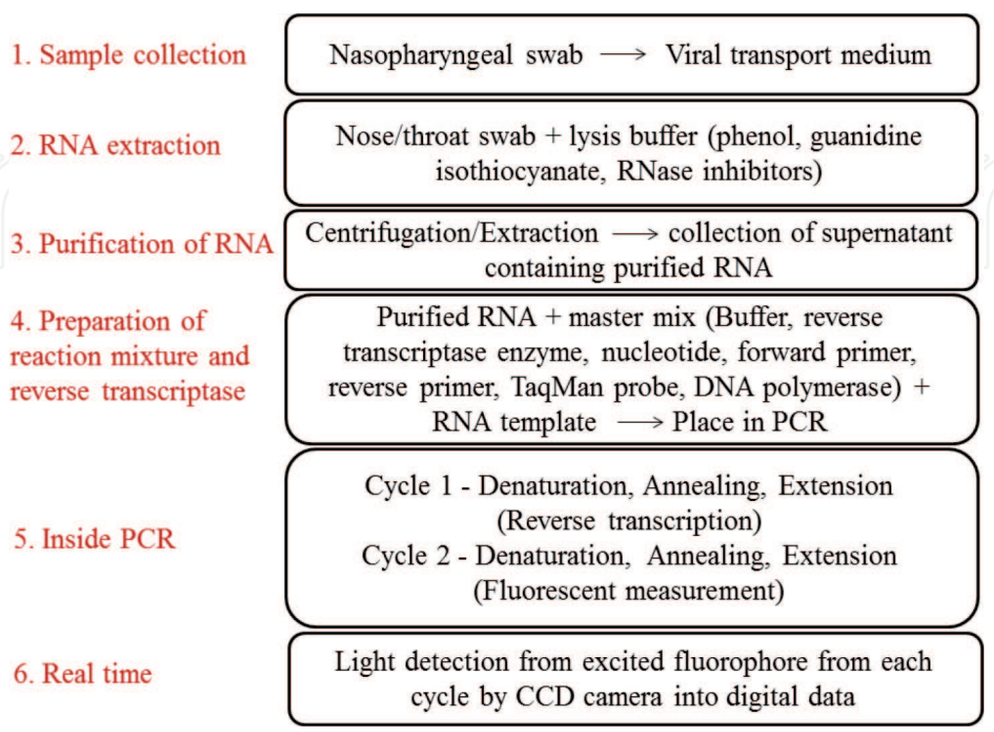


**Figure 2.**
*RT-PCR methodological performance flowchart.*

and Taq-man probe to its complementary part of the target DNA. Taq-man probe consist of a fluorophore covalentaly attached to the 5′ end of oligonucleotide probe, the fluorescence is emitted by the fluorophore when is excited by the cyclers light source. Also, this probe consists of quencher at 3′ end. The close proximity of the reporter to the quencher prevents detection of its fluorescence. When polymerase reaches the Taqman probe its endogenous 5′ nuclease activity cleaves the probe, separating the dye from the quencher, with each cycle of PCR more dyes are released, resulting in an increase in fluorescence intensity proportionately to the amount of amplicon synthesized. For the fluorescence signal a tungsten-halogen lamp, an excitation filter, lens, emission filter and charged coupled device (CCD) camera are use which converts light into digital data. A flowchart of the protocol has shown in **Figure 2** [24].

## 5. Recent development of RT-PCR for SARS-CoV-2 diagnosis

In addition to prevention methods (e.g., hygiene, social distance, isolation of infected individuals, and travel restriction), rigorous community infection testing is essential to track the transmission of the disease as well as educating public policies [53]. Nations that have implemented large research strategies at an early stage like South Korea, Vietnam, and New Zealand have been better able to restrict the spread of the disease. Tests should ideally be simple to sample and evaluate, precise, reliable, scalable and inexpensive. Often, point-of-care tests (POCT) based on antibodies match this definition. However, rapidly emerging epidemics due to novel viruses do not allow antibody-based tests to evolve in a timely manner [54]. Because of the simple adaptability to the nucleotide sequence of the target, viral load tests based on real-time, quantitative RT-PCR (referred to as RTqPCR) are thus an ideal test [55]. This RT-qPCR is currently a reliable test commonly used for the diagnosis of SARS-CoV-2 infected symptomatic and asymptomatic patients [56]. Several scientific and clinical institutions around the world have produced molecular assays to diagnose SARS-CoV-2 and have made RT-qPCR primers and sample sets available to the public [57].

As the genetic code of coronaviruses consists of RNA, its purification of the test samples is a crucial step in RT-qPCR protocols. Officially, institutions in some countries have suggested unique RNA isolation kits for SARSCoV-2 detection [57]. Various virus extraction and detections kits are available in market as shown in **Table 3**. The qRT-PCR has the benefits of high sensitivity, high precision, and a wide variety of sample types that can be use but there are however several factors that influence qRT-PCR outcomes, such as repeated washing, purification and separation of viral nucleic acids, which cause a substantial loss of nucleic acid and increase the risk of fragmentation and hydrolysis of nucleic acid; the number of virus replications below the qRT-PCR detection threshold would also cause false negative effects on the early stage of SARS-CoV-2 infection. So this problem was faced by many laboratories [58]. Some started focusing on RT-LAMP—reverse transcriptase loop-mediated isothermal amplification [59]. Other PCR, which was found to be more potent to detect low viral load was digital droplet PCR (ddPCR) as compared to qRT-PCR but due to limited availability ddPCR instrumentation and lab expertise mostly qRT-PCR is being used [60]. At this point, superior resources are concentrated in all parts of the country to reinforce scientific research. It is assumed that our ability to diagnose and treat patients with new coronavirus pneumonia will develop further with the enhancement and advancement of detection technology.

## 6. Status in India

Collection of data at one place so that whole country population can refer this data at such a tough situation was a necessity so the Government of India has open an website -https://covid19cc.nic.in/ICMR/login.aspx, which has lots of information regarding testing centers, counting of RT-PCR tests and other antibody–antigen tests, current positive cases, available collection centers with their address contacts etc. Also aligned with ICMR, said to be Covid-19 sample collection management system for rapid antigen, antibody and RT-PCR tests. Lots of RT-PCR kits with variations, are manufactured in India has been shown in **Table 4**.

| Kit | Company | Sample required | Website |
|---|---|---|---|
| Fluidigm's Advanta Dx SARS-CoV-2 RT-PCR kit | Fluidigm's Corporation | Saliva | https://www.fluidigm.com/ |
| R-Green kit (SARS COV2-real-time PCR) | Reliance Life Sciences | Throat, nose swab | https://rellife.com/ |
| Dry Swab-Direct RT-PCR test | Centre for Cellular and Molecular Biology (CCMB) | Dry Nasal swab | https://www.ccmb.res.in/ |
| RT-PCR testing kits | iGenetic Diagnostics and BioGenomics | Throat, nose swab | https://www.igenetic.com/ |
| Mylab PathoDetect COVID-19 Qualitative PCR kit | Mylab Discovery Solutions Pvt. Ltd | Throat, nose swab | https://mylabdiscoverysolutions.com/ |
| Real Time PCR kits | Genome Diagnostics Pvt. Ltd. | Nasopharyngeal swab | http://www.genomediagnostics.co.in/about-us.html |
| GlobalTM diagnostic kit | Equine Biotech | Nasopharyngeal swab | https://covid19.iisc.ac.in/covid-19-rt-pcr-kit/ |
| Corosure Covid-19 diagnostic kit | IIT Delhi | Throat, nose swab | https://home.iitd.ac.in/news-tea-haritaki.php |
| RT-PCR kit | CSIR-IICT | Throat, nose swab | https://iictindia.org/ |

**Table 4.**
*RT-PCR kit developed by Indian companies and sample requirement.*

## 7. International status

RT-qPCR assays were already known, but development can lead to better results. Thus, one study was done by targeting two monoplex assays recommended by WHO that was targeted on the envelope gene (E-Sarbeco) and RNA-Dependant RNA Polymerase coding genes (RdRp-IP4). This was combined and test named as 'Duo SARS-CoV-2 RT-qPCR assay'. As already lots of duo assays are commercially available, but this study was done to combine in-house assays. Can be used to reduce the chances of false negative results as it is dual assay [60]. As dual target assays are commercially available, developed specifically to

target multiple sites in the viral genome, but mutation rates in virus found to be moderate which can cause problem with respect to results. Some of the tests were done by using 'cobas SARS-CoV-2 E gene qRT-PCR' but some failures were seen due to change in nucleotide at position 26340 of the SARS-CoV-2 gene. So the study was done to identify single nucleotide polymorphism in E gene of the virus. This includes the importance of study regarding mutations in the virus and thus to prevent false negative results [61]. The protocol provided by WHO mention the three assay that is to be performed: First line screening assay - E gene assay, Confirmatory assay - RdRp gene assay, and Additional confirmatory assay - N gene assay [24]. 'Multiplex Real Time PCR' was also use to find its efficacy for SARS-CoV-2 detection. Found to be rapid and accurate method for viral detection [62]. 'Aus Diagnostics respiratory MT-PCR assay' was also found to be reliable and sensitive [63]. Two 'Single-tube nested (STN) real-time RT-PCR assays' specifically targets RdRp/Hel and N genes, found to be 100% specific for detection of SARS-CoV-2 [64]. Even high cost assays were given preference during a pandemic situation like TaqMan-based real-time RT-qPCR which was not even accessible to lots of laboratories. So it was performed by using 2 methods that are SYBR green RT-qPCR and conventional PCR basically standardisation was done of this 2 methods which is cost effective methods and found to give equal results as that of TaqMan-based real-time RT-qPCR [65].

COVID-19 diagnosis now done by one step RT-qPCR by using primers and probes which were developed at different institutes like China CDC, Charite (Germany), The University of Hong Kong, National Institute of Infectious Diseases in Japan (Japan NIID), National Institute of Health in Thailand (Thailand NIH) and US CDC which was announced by WHO [2, 24]. One study performed the analysis of primer-probe sets specifically targeting the N region and RdRp/Orf1 of SARS-CoV-2 by N-assay and RdRp/Orf1 Assays. Primer probe set for N-assay was N (China CDC), HKU-N (HKU), NIID_2019-nCOV_N (Japan NIID), WH-NIC N (Thailand NIH), and 2019-nCoV_N1, -N2, and -N3 (US CDC) and for RdRp/Orf1 Assay RdRp_SARSr (Charite), HKU-ORF1b-nsp14 (HKU), and ORF1ab (China CDC) primer probe set was studied. Results says that NIID_2019-nCOV_N" from the Japan NIID and "ORF1ab" from China CDC gave a good performance for RT-qPCR analysis without any cross-reactivity and non-specific amplifications. This can be used for further diagnosis [66]. Sensitivity of PCR also depends on primer concentration, degeneration and multi target detection. Initial concentration of primer was 300-900 nM but as its concentration rises sensitivity also improves [67]. One study found that concentration upto 400 nM rise the sensitivity [68]. Degenerate primers plays an important role with respect to diversity of SARS-CoV-2. While screening assays with a single target area are more prone to sequence differences than dual or triple-target assays for multi-target identification [67]. Other than this primer length, melting temperature, GC content and annealing temperature also affects the sensitivity of PCR assay [67]. One study attempted to deduce the specific patterns among SARS-CoV-2 isolates and accordingly primers were design targeted to nsp2 gene and further use for diagnosis of probe free real-time RT-PCR. Sensitive and rapid SARS-CoV-2-specific real-time RT-PCR assay COVID-19-nsp2 has therefore been developed [69]. In one research, the efficiency of three novel real-time reverse transcription-PCR (RT-PCR) assays targeting RNA-dependent RNA polymerase (RdRp)/helicase (Hel), spike (S) and nucleocapsid (N) SARS-CoV-2 genes was developed and compared. RNA polymerase (RdRp)/(Hel) assay found to be effective with no cross reactivity with other viruses among samples [27]. Some of the RT-PCR kits manufactured at International level along with sample requirement has been shown in **Table 5**.

| Kit Name | Company | Country | Sample required | Website |
|---|---|---|---|---|
| 1copy™ COVID-19 qPCR Multi Kit | 1drop Inc. | Korea | Nasal swab | http://www.1drop.co.kr/ |
| ANDiS® SARS-CoV-2 RT-qPCR Detection Kit | 3D Medicines | United state | Nasopharyngeal swab | https://www.3dmedcare.com/covid/ |
| CareStart™ COVID-19 MDx RT-PCR | Access Bio, Inc. | United state | Nasopharyngeal swab, Oropharyngeal swab | https://carestart.com/ |
| MOLgen SARSCoV2 Real Time RT PCR | Adaltis S.r.l. | Italy | Nasopharyngeal swabs, oropharyngeal swabs, sputum and bronchoalveolar lavage fluid (BALF). | http://www.adaltis.net/products/molecular-diagnostic-tests/sars-cov2-covid-19/sars-cov2-covid-19/ |
| LyteStar 2019-nCoV RT-PCR Kit 1.0 | ADT Biotech | Malaysia | Nasopharyngeal swab, Oropharyngeal swab | http://adt-biotech.com/lytestartm-detection-kits/ |
| AccuPower® SARS-CoV-2 Real-Time RT-PCR kit | BIONEER Corporation | Philippines | Sputum, nasopharyngeal swab, oropharyngeal swab | https://eng.bioneer.com/20-scv-2122.html |
| VIASURE SARS-CoV-2 S gene Real Time PCR Detection Kit adapted for BD MAX™ System | CerTest Biotec, S.L. | Spain | Respiratory samples | https://www.certest.es/ |
| Simplexa™ COVID-19 Direct RT-PCR Kit | DiaSorin Molecular, LLC | United state | Nasal swab, nasopharyngeal swab, nasal wash/aspirate, and BAL specimens | https://molecular.diasorin.com/us/kit/simplexa-covid-19-direct-kit/ |
| Quick SARS-CoV-2 rRT-PCR Kit | Zymo Research Corp. | United state | Upper respiratory and lower respiratory sample | https://www.zymoresearch.com/ |
| SARS-CoV-2 Nucleic Acid Detection Kit (PCR-Fluorescent Probe Method) | Zybio, Inc. | Philippines | Nasal/throat swab, bronchoalveolar lavage fluids, stool | https://m.zybio.com/en/Product/Molecule/2020-03-24/316.html |
| Novel Coronavirus (2019-nCoV)/ Flu A/Flu B Real-time Multiplex RT-PCR Kit | Zhuhai Haitai Biological Pharmaceutical Co., Ltd | China | Upper respiratory and lower respiratory sample | http://www.zhhaitai.com/ |
| MolecuTech® Real-Time COVID-19 | YD Diagnostics Corp. | Korea | Respiratory specimens | http://www.yd-diagnostics.com/2012/eng/channel_02/prt_list.php?selID=37 |

| Kit Name | Company | Country | Sample required | Website |
|---|---|---|---|---|
| COVID-19 ORF1ab/N Gene PCR Detection Kit | Xi'an Tianlong Science and Technology Co., Ltd | China | Upper respiratory and lower respiratory sample | https://xi-an-tianlong.abraa.com/ |
| Real time RT-PCR Kit for the detection of SARS-CoV-2 | Suzhou BTA Biotech Co. Ltd | China | Nasal swab, nasopharyngeal swab, nasal wash/aspirate, and BAL specimens | http://vosunbio.com/ |
| LyoDx® A Freeze-Dried Real-Time RT-PCR Detection Reagent for SARS-CoV-2 | SignalDT Biotechnologies (SZ), Inc. | Alameda | Respiratory samples | https://signaldt-biosystems-llc.hub.biz/ |
| SBC SARS-CoV-2 Convective PCR Diagnostic Device/Kit | Schweitzer Biotech Company Ltd | Taiwan | Nasopharyngeal or oropharyngeal swabs | http://covid19.sbc-biotech.com/ |
| RainSure COVID-19 dPCR Detection Kit (lab-based) | RainSure Scientific Co., Ltd | China | Respiratory samples | http://en.rainsurebio.com/ |
| COVID-19 One-Step COVID-19 RT-PCR Kit | Pishtaz Teb Diagnostics | Iran | Nasopharyngeal or oropharyngeal swabs | https://pishtazteb.com/en/pcr/ |
| PerkinElmer® SARS-CoV-2 Realtime RT-PCR Assay | PerkinElmer Inc. | United state | Nasopharyngeal or oropharyngeal swabs | https://www.perkinelmer.com/ |
| SARS-CoV-2 One-Step RT-PCR Kit, RdRp and N Genes, CE-IVD | NZYTech | Portugal | Nasal swab, nasopharyngeal swab, nasal wash/aspirate, and BAL specimens | https://www.nzytech.com/products-services/molecular-diagnostics/sars-cov-2/sars-cov-2-one-step-rt-pcr-kit-rdrp-and-n-genes-ce-ivd/ |
| COVID-19 TaqMan RT-PCR Kit (E/RdRP genes) Dx | Norgen Biotek Corp | Canada | Saliva & Swab Samples | https://norgenbiotek.com/ |

**Table 5.**
*RT-PCR kits developed by International companies and sample requirement.*

## 8. Future prospects of RT-PCR

PCR method was discovered in 1986 and since then the method is serving medical sectors. In the future, as potential molecular diagnostic methods, PCR will play a significant role. Lots of PCR methods are already used in various research and medical fields, but as we know currently under this pandemic situation RT-PCR has turned out to be a boon in healthcare sectors. Lots of kits have been manufactured throughout the world with some or little variation, thus making it more sensitive, specific and less time consuming. One factor which essential under pandemic situation was a multiple sample analysis in one go [5]. Thus more focus on using portable POC systems, we can imagine the use of micro-fluidics doing concurrent multiple sample analysis. It is possible to analyse immunomagnetic exosomal RNA by using micro-fluidic systems RT-PCR said to be a Chip-based integrated real-time reverse transcription PCR platform [70]. Similar way chip-based RT-PCR digitally can quantify the mRNA in single cell [71].

Scalable, quick, and inexpensive diagnostics of COVID-19 by RT-PCR could help restrict the spread of SARS-CoV-2, saving lives as a result. RNA extraction, however, constitutes an obstacle to the scale-up of experiments. Thus, one research was done directly using the RT-PCR and heat inactivated sample and efficacy was tested. The study proved that is not necessary to carry RNA extraction testing. The study also suggests the use of standard protocols for RT-PCR and transport media by the whole world so the it will become easier to deal with future epidemics. Such RT-PCR said to be an hid RT-PCR (heat-inactivated direct RT-PCR). Sample collection and rather than using transport media, usage of lysis buffer is found to be more efficient as it will directly lys the sample and can be used in RT-PCR without a need of any RNA extraction kit [72].

As *Coronaviridae* family consist many RNA viruses which not only infect the humans but also animals, birds etc. [73]. If we look at the history of this family will see that it has been always serious when it started infecting like in 2002, Severe acute respiratory syndrome coronavirus (SARS-CoV) emerged, in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV), in 2016, swine acute diarrhoea syndrome coronavirus (SADS-CoV) and the latest one which cause pandemic in 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [74]. Among this SADS-CoV infects pigs while other infect humans [75]. This all was controlled well but current 2019 epidemic was beyond control due to lack of diagnosis, medicines and vaccine. For now after all efforts diagnosis for SARS-CoV-2 has been found but as lots of virus under this family can emerge in future and which has already spread in past needed to be studied [76]. So one research was completely done to make a molecular diagnostic kit which can identify almost all CoVs, thus to diagnose easily any virus among this family in future [77]. They designed a semi-nested RT-PCR based upon 38 genome sequences that has been recorded from human and animal CoVs. Thus proved to be a great finding which can diagnose all available CoVs or which can emerge in future too [77].

Lots of research regarding RT-PCR has been done, PCR like nested PCR, ddPCR, two step RT-PCR is an advance and gives more accurate results but labs were not that equipped and it was not possible to set up everything under such pandemic situation. Hence if we think about future this all equipments and facilities have to be adopted by laboratory to deal with emerging epidemics [57].

## 9. Conclusion

SARs-CoV-2, originated from Wuhan, China and spread all over world, causing a pandemic situation which affected the whole world badly at economic, social,

medical level also. Initially it was very difficult to deal with virus as no diagnosis, treatment or vaccine was available, but after lots of efforts of researchers now we have a good diagnosis and control condition in regards spread of infection. Even vaccination has been started in almost all countries. As in the earlier period of pandemic, the diagnosis was the main factor to prevent the spread of the virus. So main focus was on a diagnostic that to on molecular diagnosis as it is more efficient and accurate way of detection. Currently use molecular method is RT-PCR also said to be a gold standard detection method. Lots of RT-PCR kits are now available in most of the countries with little modifications and approvals. The key point to be noticed before the laboratory experiments is the right reliable sampling. Nasopharyngeal and oropharyngeal swabs, which are safer for collection are recommended for screening or early detection. If we talk about a future of RT-PCR advancement in methods like nested PCR, ddPCR, two step RT-PCR have been already done, which is found to be more accurate, but lack of instrumentation and expertise have put it behind, but in future this thing has to be focused and implemented so that the world can deal with any future epidemic.

## Author details

Rutuja Sunil Patankar[1] and Vasudeo Pandharinath Zambare[1,2*]

1 School of Science, Sandip University, Nashik, India

2 R&D Department, Om Biotechnologies, Nashik, India

*Address all correspondence to: vasuzambare@gmail.com

## References

[1] Christensen, J., Senthilingam, M. (2020) CNN https://edition.cnn.com/2020/01/20/health/whatis-coronavirus explained/index.html.

[2] WHO (2020a) 'Cambodia: WHO strengthens response to COVID-19 with support from the World Bank and UN'https://www.who.int/news-room/feature stories/detail/cambodia-who-strengthens-response-to-covid-19-with-support-from-the-world-bank-and-un.

[3] H. A. Rothan and S. N. Byrareddy, "The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak," J. Autoimmun., vol. 109, no. February, p. 102433, 2020, doi: 10.1016/j.jaut.2020.102433.

[4] W. Zhao, J. Zhang, M. E. Meadows, Y. Liu, T. Hua, and B. Fu, "A systematic approach is needed to contain COVID-19 globally," Sci. Bull., vol. 65, no. 11, pp. 876-878, 2020, doi: 10.1016/j.scib.2020.03.024.

[5] H. Zhu, H. Zhang, Y. Xu, S. Laššáková, M. Korabečná, and P. Neužil, "PCR past, present and future," Biotechniques, vol. 69, no. 4, pp. 317-325, 2020, doi: 10.2144/BTN-2020-0057.

[6] W. Zhao, J. Zhang, M. E. Meadows, Y. Liu, T. Hua, and B. Fu, "A systematic approach is needed to contain COVID-19 globally," Sci. Bull., vol. 65, no. 11, pp. 876-878, 2020, doi: 10.1016/j.scib.2020.03.024.

[7] X. Ren et al., "Application and Optimization of RT-PCR in Diagnosis of SARS-CoV-2 Infection," SSRN Electron. J., 2020, doi: 10.2139/ssrn.3546086.

[8] L. J. Carter et al., "Assay Techniques and Test Development for COVID-19 Diagnosis," ACS Cent. Sci., vol. 6, no. 5, pp. 591-605, 2020, doi: 10.1021/acscentsci.0c00501.

[9] D. K. W. Chu et al., "Molecular Diagnosis of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia," Clin. Chem., vol. 7, pp. 1-7, 2020, doi: 10.1093/clinchem/hvaa029.

[10] J. A. Porcel-Vazquez et al., "Usefulness of PCR Screening in the Initial Triage of Trauma Patients During COVID-19 Pandemic," J. Orthop. Trauma, vol. 34, no. 10, pp. e377–e381, 2020, doi: 10.1097/BOT.0000000000001903.

[11] P. Freire, Byron et al. 2020. "Cotton-Tipped Plastic Swabs for SARS-CoV-2 RT-QPCR Diagnosis to Prevent Supply Shortages." Frontiers in Cellular and Infection Microbiology 10(June): 1-4.

[12] A. Sowood et al., "Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID- 19 . The COVID-19 resource centre is hosted on Elsevier Connect , the company ' s public news and information ," no. January, 2020.

[13] J. Alcoba-Florez et al., "Fast SARS-CoV-2 detection by RT-qPCR in preheated nasopharyngeal swab samples," Int. J. Infect. Dis., vol. 97, pp. 66-68, 2020, doi: 10.1016/j.ijid.2020.05.099.

[14] M. R. Hasan et al., "Detection of SARS-CoV-2 RNA by direct RT-qPCR on nasopharyngeal specimens without extraction of viral RNA," PLoS One, vol. 15, no. 7 July, pp. 1-9, 2020, doi: 10.1371/journal.pone.0236564.

[15] N. Lübke et al., "Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID- 19 . The COVID-19 resource centre is hosted on Elsevier Connect , the company ' s public news and information ," no. January, 2020.

[16] E. González-González, G. Trujillo-De Santiago, I. M. Lara-Mayorga, S. O. Martínez-Chapa, and M. M. Alvarez, "Portable and accurate diagnostics for COVID-19: Combined use of the miniPCR thermocycler and a well-plate reader for SARS-CoV-2 virus detection," PLoS One, vol. 15, no. 8 August, pp. 1-13, 2020, doi: 10.1371/journal.pone.0237418.

[17] S. Klein et al., "SARS-CoV-2 RNA extraction using magnetic beads for rapid large-scale testing by RT-qPCR and RT-LAMP," Viruses, vol. 12, no. 8, 2020, doi: 10.3390/v12080863.

[18] S. Srivatsan et al., "Preliminary support for a 'dry swab, extraction free' protocol for SARS-CoV-2 testing via RT-qPCR," bioRxiv Prepr. Serv. Biol., pp. 1-2, 2020, doi: 10.1101/2020.04.22.056283.

[19] P. B. van Kasteren et al., "Comparison of seven commercial RT-PCR diagnostic kits for COVID-19," J. Clin. Virol., vol. 128, no. April, p. 104412, 2020, doi: 10.1016/j.jcv.2020.104412.

[20] X. Wang et al., "Limits of Detection of 6 Approved RT-PCR Kits for the Novel SARS-Coronavirus-2 (SARS-CoV-2)," Clin. Chem., vol. 66, no. 7, pp. 977-979, 2020, doi: 10.1093/clinchem/hvaa099.

[21] K. Ramanathan et al., "Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding," Lancet, vol. 2020, no. January, pp. 19-21, 2020.

[22] D. K. W. Chu *et al.*, "Molecular Diagnosis of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia," Clin. Chem., vol. 7, pp. 1-7, 2020, doi: 10.1093/clinchem/hvaa029.

[23] Center for Disease Control, "Research Use Only 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Primers and Probes," *CDC's Diagnostic Test COVID-19 Only Supplies*, vol. 19, pp. 2019-2020, 2019, [Online]. Available: https://www.cdc.gov/coronavirus/2019-ncov/lab/virus-requests.html.

[24] V. M. Corman *et al.*, "Detection of 2019 -nCoV by RT-PCR," Euro Surveill, vol. 25, no. 3, pp. 1-8, 2020.

[25] J. F. W. Chan *et al.*, "A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster," Lancet, vol. 395, no. 10223, pp. 514-523, 2020, doi: 10.1016/S0140-6736(20)30154-9.

[26] N. Younes et al., "Challenges in Laboratory Diagnosis of the Novel," Mdpi, pp. 1-27, 2020.

[27] S. J. R. Da Silva et al., "Clinical and Laboratory Diagnosis of SARS-CoV-2, the Virus Causing COVID-19," ACS Infect. Dis., vol. 6, no. 9, pp. 2319-2336, 2020, doi: 10.1021/acsinfecdis.0c00274.

[28] Arevalo-Rodriguez et al., "False-Negative Results of Initial Rt-Pcr Assays for Covid-19: a Systematic Review," pp. 1-33, 2020, doi: 10.1101/2020.04.16.20066787.

[29] J. Watson, P. F. Whiting, and J. E. Brush, "Interpreting a covid-19 test result," BMJ, vol. 369, no. May, pp. 1-7, 2020, doi: 10.1136/bmj.m1808.

[30] J. A. Porcel-Vazquez et al., "Usefulness of PCR Screening in the Initial Triage of Trauma Patients During COVID-19 Pandemic," J. Orthop. Trauma, vol. 34, no. 10, pp. e377–e381, 2020, doi: 10.1097/BOT.0000000000001903.

[31] Jamie Ducharme (2020) 'What to Know About COVID-19 Tests, From PCR to Antigen to Antibody'

https://time.com/5880255/covid-19-tests-types/.

[32] Michael Greenwood (2020) 'Quick and affordable saliva-based COVID-19 test developed by Yale scientists receives FDA Emergency Use Authorization' https://news.yale.edu/2020/08/15/yales-rapid-covid-19-saliva-test-receives-fda-emergency- use-authorization.

[33] Kaunain Sheriff M (2020) 'Explained: How rapid antigen tests detect Covid-19, where they will be used in India' https://indianexpress.com/article/explained/how-rapid-antigen-test-detects- coronavirus-where-it-will-be-used-6460735/.

[34] WION (2020) 'Delhi: 11% symptomatic negative tests of RAT found positive via RT-PCR'https://www.wionews.com/india-news/delhi-11-symptomatic-negative-tests-of-rat-found-positive-via-rt-pcr-348153.

[35] Ahmedabad Mirror (2020) 'RAT is out of the bag. Take Covid test again' https://ahmedabadmirror.indiatimes.com/ahmedabad/cover-story/rat-is-out-of-the-bag-take-covid-test-again/articleshow/78048993.cms.

[36] I. A. Mattioli and F. N. Crespilho, "Problems of interpreting diagnostic tests for SARS-CoV-2: Analytical chemistry concerns," An. Acad. Bras. Cienc., vol. 92, no. 4, pp. 1-3, 2020, doi: 10.1590/0001-3765202020201208.

[37] B. C. Behera, R. R. Mishra, and H. Thatoi, "Recent biotechnological tools for diagnosis of corona virus disease: A review," Biotechnol. Prog., no. August, pp. 1-16, 2020, doi: 10.1002/btpr.3078.

[38] E. C. Stites and C. B. Wilen, "The Interpretation of SARS-CoV-2 Diagnostic Tests," Med, vol. 1, no. 1, pp. 78-89, 2020, doi: 10.1016/j.medj.2020.08.001.

[39] Y. Tang, J. E. Schmitz, D. H. Persing, and C. W. Stratton, "crossm Challenges," J. Clin. Microbiol., vol. 58, no. 6, pp. 1-9, 2020.

[40] B. A. Parikh, T. C. Bailey, P. G. Lyons, and N. W. Anderson, "The brief case: 'Not Positive' or 'Not Sure'—COVID-19-negative results in a symptomatic patient," J. Clin. Microbiol., vol. 58, no. 8, pp. 1-6, 2020, doi: 10.1128/JCM.01195-20.

[41] J. Wang et al., "Novel One-Step Single-Tube Nested Quantitative Real-Time PCR Assay for Highly Sensitive Detection of SARS-CoV-2," Anal. Chem., vol. 92, no. 13, pp. 9399-9404, 2020, doi: 10.1021/acs.analchem.0c01884.

[42] M. Crippa, G. B. Bartolucci, F. Toffoletto, and G. Marcer, "Occupational diseases due to allergic and toxic chemicals in health care workers: Fitness for work," Med. del Lav., vol. 103, no. 3, pp. 187-197, 2012.

[43] R. Kumar, S. Nagpal, S. Kaushik, and S. Mendiratta, "COVID-19 diagnostic approaches: different roads to the same destination," VirusDisease, vol. 31, no. 2, pp. 97-105, 2020, doi: 10.1007/s13337-020-00599-7.

[44] W. Zhang et al., "Molecular and serological investigation of 2019-nCoV infected patients: implication of multiple shedding routes," Emerg. Microbes Infect., vol. 9, no. 1, pp. 386-389, 2020, doi: 10.1080/22221751.2020.1729071.

[45] M. Arya, I. S. Shergill, M. Williamson, L. Gommersall, N. Arya, and H. R. H. Patel, "Basic principles of real-time quantitative PCR," Expert Rev. Mol. Diagn., vol. 5, no. 2, pp. 209-219, 2005, doi: 10.1586/14737159.5.2.209.

[46] Y. Li et al., "Stability issues of RT-PCR testing of SARS-CoV-2

for hospitalized patients clinically diagnosed with COVID-19," J. Med. Virol., vol. 92, no. 7, pp. 903-908, 2020, doi: 10.1002/jmv.25786.

[47] A. Piras, D. Rizzo, S. Uzzau, G. De Riu, S. Rubino, and F. Bussu, "Inappropriate Nasopharyngeal Sampling for SARS-CoV-2 Detection Is a Relevant Cause of False-Negative Reports," Otolaryngol. - Head Neck Surg. (United States), vol. 163, no. 3, pp. 459-461, 2020, doi: 10.1177/0194599820931793.

[48] A. Piras et al.,Nasopharyngeal swab collection in the suspicion of Covid-19," no. January, 2020.

[49] A. Nasal et al., "S h e e t S A R S - C o V - 2 ( C O V I D - 1 9 ) F a c t Anterior Nasal ( Nares ) Specimen Collection for SARS-CoV-2 Diagnostic Testing," no. 6.

[50] CDC, "Preparation of Viral Transport Medium," Centers Dis. Control Prev., vol. 36, no. 4, pp. 268-269, 2004.

[51] U. Jan, "COVID-19 Interim Guidelines for Collecting , Handling , and Testing Clinical Specimens for COVID-19 Summary of Recent Changes Updates as of December 30 , 2020," pp. 1-6, 2021.

[52] G. P. Rédei, "RNA Extraction," Encycl. Genet. Genomics, Proteomics Informatics, pp. 1723-1723, 2008, doi: 10.1007/978-1-4020-6754-9_14747.

[53] P. G. Walker, C. Whittaker, and O. Watson, "Report 12 - The global impact of COVID-19 and strategies for mitigation and suppression | Faculty of Medicine | Imperial College London," vol. 071, no. May, 2020, doi: 10.25561/77735.

[54] S. Report, "COVID 19: Facts and Insights, October 30," pp. 1-142, 2021.

[55] Z. D. Tong *et al.*, "Potential presymptomatic transmission of SARS-CoV-2, Zhejiang Province, China, 2020," Emerg. Infect. Dis., vol. 26, no. 5, pp. 1052-1054, 2020, doi: 10.3201/eid2605.200198.

[56] C. Rothe *et al.*, "Transmission of 2019-nCoV Infection from an Asymptomatic Contact in Germany," N. Engl. J. Med., vol. 382, no. 10, pp. 970-971, 2020, doi: 10.1056/nejmc2001468.

[57] H. G. Barreto, F. A. de Pádua Milagres, G. C. de Araújo, M. M. Daúde, and V. A. Benedito, "Diagnosing the novel SARS-CoV-2 by quantitative RT-PCR: variations and opportunities," J. Mol. Med., vol. 98, no. 12, pp. 1727-1736, 2020, doi: 10.1007/s00109-020-01992-x.

[58] B. Xiao, Q. Zhou, T. Lei, Y. He, and L. Li, "Application of laboratory diagnostic technologies for SARS-CoV-2: current progress and prospect," *Nan Fang Yi Ke Da Xue Xue Bao*, vol. 40, no. 4, pp. 601-605, 2020, doi: 10.12122/j.issn.1673-4254.2020.04.24.

[59] M. F. Österdahl *et al.*, "Detecting SARS-CoV-2 at point of care: preliminary data comparing loop-mediated isothermal amplification (LAMP) to polymerase chain reaction (PCR)," BMC Infect. Dis., vol. 20, no. 1, pp. 1-8, 2020, doi: 10.1186/s12879-020-05484-8.

[60] F. Yu *et al.*, "Quantitative detection and viral load analysis of SARS-CoV-2 in infected patients," Clin. Infect. Dis., vol. 71, no. 15, pp. 793-798, 2020, doi: 10.1093/cid/ciaa345.

[61] Pezzi, Laura et al. 2020. "Development and Evaluation of a Duo SARS-CoV-2 RT-QPCR Assay Combining Two Assays Approved by the World Health Organization Targeting the Envelope and the RNA-Dependant RNA Polymerase (RdRp) Coding Regions." Viruses 12(6): 1-12.

[62] Utilized, Transcription-pcr. 2020. "Crossm A Recurrent Mutation at Position 26340 of SARS-CoV-2 Is

Associated with Failure of the E Gene Quantitative Reverse." 58(10): 1-8.

[63] Hoffmann, Carly A et al. 2020. "Ac Ce Pt e d an Us Cr Ip t Ac Ce Pt e Us Cr." : 1-27.

[64] Attwood, Lucy O et al. 2020. "Since January 2020 Elsevier Has Created a COVID-19 Resource Centre with Free Information in English and Mandarin on the Novel Coronavirus COVID-19 . The COVID-19 Resource Centre Is Hosted on Elsevier Connect , the Company ' sPublic News and Information ." (January).

[65] Dorlass, Erick Gustavo et al. 2020. "Lower Cost Alternatives for Molecular Diagnosis of COVID-19: Conventional RT-PCR and SYBR Green-Based RT-QPCR." Brazilian Journal of Microbiology 51(3): 1117-1123.

[66] Jung et al., "Comparative Analysis of Primer-Probe Sets for RT-qPCR of COVID-19 Causative Virus (SARS-CoV-2)," ACS Infect. Dis., vol. 6, no. 9, pp. 2513-2523, 2020, doi: 10.1021/acsinfecdis.0c00464.

[67] Li, Dandan, Jiawei Zhang, and Jinming Li. 2020. "Primer Design for Quantitative Real-Time PCR for the Emerging Coronavirus SARS-CoV-2." Theranostics 10(16): 7150-7162.

[68] Vijgen, Leen et al. 2008. "A Pancoronavirus RT-PCR Assay for Detection of All Known Coronaviruses." Methods in Molecular Biology 454: 3-12.

[69] Yip, Cyril Chik Yan, Siddharth Sridhar, et al. 2020. "Development and Evaluation of Novel and Highly Sensitive Single-Tube Nested Real-Time Rt-Pcr Assays for Sars-Cov-2 Detection." International Journal of Molecular Sciences 21(16): 1-11.

[70] H. Shao *et al.*, "Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma," Nat. Commun., vol. 6, p. 25959588, 2015, doi: 10.1038/ncomms7999.

[71] A. M. Thompson, A. Gansen, A. L. Paguirigan, J. E. Kreutz, J. P. Radich, and D. T. Chiu, "Self-digitization microfluidic chip for absolute quantification of mRNA in single cells," Anal. Chem., vol. 86, no. 24, pp. 12308-12314, 2014, doi: 10.1021/ac5035924.

[72] I. Smyrlaki *et al.*, "Massive and rapid COVID-19 testing is feasible by extraction-free SARS-CoV-2 RT-PCR," Nat. Commun., vol. 11, no. 1, pp. 1-12, 2020, doi: 10.1038/s41467-020-18611-5.

[73] P. Zhou *et al.*, "A pneumonia outbreak associated with a new coronavirus of probable bat origin," Nature, vol. 579, no. 7798, pp. 270-273, 2020, doi: 10.1038/s41586-020-2012-7.

[74] J. Guarner, "Three Emerging Coronaviruses in Two Decades: The Story of SARS, MERS, and Now COVID-19," Am. J. Clin. Pathol., vol. 153, no. 4, pp. 420-421, 2020, doi: 10.1093/ajcp/aqaa029.

[75] P. Zhou *et al.*, "Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin," Nature, vol. 556, no. 7700, pp. 255-259, 2018, doi: 10.1038/s41586-018-0010-9.

[76] D. P. Oran and E. J. Topol, "Prevalence of Asymptomatic SARS-CoV2 Infection : A Narrative Review," Ann.Intern. Med., vol. 173, no. 5, pp. 362-367, 2020, doi: 10.7326/M20-3012.

[77] L. Xiu *et al.*, "A RT-PCR assay for the detection of coronaviruses from four genera," J. Clin. Virol., vol. 128, pp. 1-2, 2020, doi: 10.1016/j.jcv.2020.104391.