MINI REVIEW

RT-PCR for COVID-19: Diagnosis Made Easy

Osheen Sajjad[1], Aiman Shahzad[2], Saqib Mahmood[3]

ABSTRACT

Coronavirus disease COVID-19, caused by Severe Acute Respiratory Syndrome Corona Virus-2 (SARS-CoV-2), is highly contagious and has been a pandemic since March 2020. The SARS-CoV-2 is an enveloped, single-stranded, positive-sense RNA viruswhich spreadsthrough air droplets by sneezing and coughing from affected person. The diagnosis of the COVID-19 remains a challenge to the scientists since the genome of the SARS-CoV-2 was novel and varying. Various studies have reported the validated procedures for sampling and the detection method of SARS-CoV-2. This mini-review provides a brief introduction of the SARS-CoV-2 features and the current knowledge for the recommended COVID19 detection methods including sampling procedures and real time SARS-CoV-2 genome detection.

KEYWORDS: RNA extraction, Real time-PCR**,** Diagnosis, COVID-19.

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INTRODUCTION

In December 2019, an outbreak was identified in the city of Wuhan, China and its rapid spread contributed to the decision of World Health Organization (WHO) to declare it a public health emergency in January, 2020 and as pandemic in March, 2020. The virus responsible for this outbreak was identified and named Severe Acute Respiratory Syndrome Corona Virus-2 (SARS-CoV-2). Subsequently the disease caused by this virus was named Coronavirus Disease 2019 (COVID-19) by WHO. The viral genome sequence revealed that SARS-CoV-2 is a member of the genus *Betacoronavirus*, belonging to subgenus, *Sarbecovirus*. Coronaviruses are enveloped, single-stranded, positive-sense RNA viruses. Positive-sense RNA means that the genomic RNA is directly translated into proteins without forming intermediate mRNA. In the past, the viruses of this family were responsible for outbreaks of diseases like Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). COVID-19 is highly contagious and the mode of spread is through droplets by sneezing or coughing from affected persons.[1] The clinical manifestations of the COVID-19 patients include radiological evidence of pneumonia, high grade fever, nonproductive cough, dyspnea, fatigue, normal or decreased leukocyte counts.[2] It has been estimated that 2% of the population is asymptomatic carriers of the virus and almost 5% to 10% suffer from acute respiratory infections.[3] SARS-CoV-2 genome consists of the 29891 nucleotides, encoding for 9860 amino acids.[4] The viral replicase transcriptase complex is encoded by the 5'- terminal; two-thirds of the viral genome encodes non-structural proteins, which is likely to be involved in the transcription and replication of the virus. Four main structural proteins: spike (S), envelope (E), nucleo-capsid (N) and membrane (M) proteins, as

1. Osheen Sajjad
2. Aiman Shahzad
3. Saqib Mahmood

1-3 Institute of Biomedical and Allied Health Sciences
University of Health Sciences, Lahore– Pakistan.

Corresponding Author:
Dr. Saqib Mahmood
Head, Institute of Biomedical and Allied Health Sciences
University of Health Sciences, Lahore– Pakistan.
Email: medgen@uhs.edu.pk

well as several accessory proteins are encoded by the other one-third of the genome[5] as shown in the Figure.1. The Spike protein of the CoV-2 determines the virus entry into the host cells by binding to the Angiotensin converting enzyme-2 (ACE2) receptor on the host cell.[6]

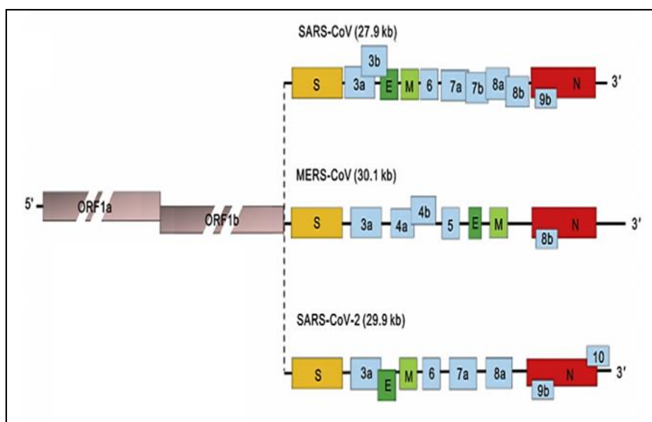

Fig.1: CoV-2 RNA genome structure comparison with other coronaviruses.[6]

Diagnosis of Cov-2 cannot be made on the clinical presentation alone as the symptoms including fever, cough, fatigue, sputum production and shortness of breath are non-specific symptoms and can be found in other conditionstoo. Molecular testing is the most reliable laboratory technique recommended by World Health Organization (WHO) and is currently the gold standard for diagnosis of COVID-19. Real-time quantitative polymerase chain reaction (RT-PCR) and high-throughput sequencing are being currently used for the molecular detection of the SARS-CoV-2. Virus containing blood culture is considered to be the reliable identification of the SARS-CoV-2 and the high-throughput sequencing of the whole genome is the gold standard detection method but it cannot be used for the COVID-19 diagnostic purpose due to its equipment dependency and high cost. Therefore, the most common, reliable, efficient and cost-effective method for the detection of SARS-CoV-2 is rRT-PCR.[7]

In rRT-PCR, SARS-CoV-2 RNA genome is first converted into complementary DNA (cDNA) strands and then the specific regions of the cDNA are amplified exponentially by PCR. The diagnosis can then be made by identifying the threshold cycle (Ct value) of the qRT-PCR at which the detectable copies of the targeted region of virus have been amplified.

Sampling Procedures

Fast and accurate results from laboratory testing are the result of good sampling procedures in case of SARS-CoV-2. For the detection of SARS-CoV-2, like other respiratory viruses, sample can be taken from upper (nasopharyngeal (NP) or oropharyngeal (OP) swabs or saliva) or lower respiratory tract, sputum or tracheal aspirate or Broncho-alveolar lavage (BAL), in symptomatic or severe cases. SARS-CoV-2replicates in the upper respiratory tract which peaks at day five of infection ofSARS-CoV-2. Thetime duration of infection and the anatomical site of sampling correspondence is an important factor to reduce the false negatives results which is a continuous issue being faced by the molecular diagnostic team of SARS-CoV-2.Sampling from asymptomatic or mild cases is an important part of diagnosis as the specimen taken from the upper respiratory tract of these patients may show false negative results; repeat sampling from multiple sites is therefore needed.[8]

Yang and his colleagues[9] have recommended taking samples from different anatomical sites at different intervals of the infection starting from the day zero to more than 15 days for mild or severe patients. In the first week of infection sputum is most accurate, followed by nasal swabs. Throat swabs are not recommended in diagnosing COVID-19 during the second week of infection and after the second week, bronchioalveolar lavage (BAL) is recommended for diagnosis and monitoring in severe cases.[9] Some believe that NP or OP can cause sneezing or coughing during sampling and may release viral aerosols therefore throat samples and saliva are preferred over NP for more comfort, non-invasive, less health care worker exposure.[8]But Centre for Disease Control (CDC) recommends only the NP sample with synthetic fiber swabs having plastic or wire shafts, but the OP swabs specimencan also be used. It is recommended if the specimen has been taken from both the sides i.e. NP and OP, then they should be collected in a single tube which will increase the sensitivity of the test by limiting the use of resources.

Minitip of nasal swab is inserted through the nostril in a direction parallel to the palate. Minitip is inserted to the distance in the nostril equivalent of that side of ear to the nostril of the patient. The resistance encountered is the indication for the contact of minitip with the nasopharynx.[10]It is gently rubbed and rolled and left for several seconds to absorb secretions. Sample from both the sides can be taken if the minitip is not saturated with the fluid or there remains difficulty from taking sample due to deviated septum or blockage on one nostril as shown in the Figure.2. For the oropharyngeal sample, swab is inserted into the posterior pharynx and tonsillar areas and rubbed over both tonsillar pillars and posterior oropharynx. It should be done carefully so the swab may not touch with the tongue, teeth, and gums in order to avoid contamination by other residing microorganisms.

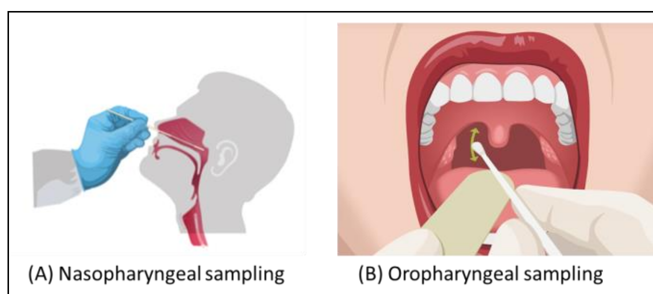

Fig.2: Nasopharyngeal and Oropharyngeal sampling for COVID-19.

Swabs are immediately placed into a sterile transport tube containing 2 – 3 mL of either viral transport medium (VTM), Amies transport medium, or sterile saline. VTM contains Hanks Balanced Salt Solution, heat-inactivated fetal bovine serum (FBS), Gentamicin sulfate (an antibiotic) and Amphotericin B (an antifungal).[11] Specimens are then stored at 2 – 8°C for up to 72 hours after collection and if a delay in testing or shipping is expected, it is stored at -70°C or below.[12]

RNA Extraction

Specimen handling for molecular testing would require BSL-2 or equivalent facilities.[13] Working in the BSL-2 lab for CoV-2 detection requires tyevak suits, surgical masks, N95 masks, gloves, eye protecting goggles, face shields, booties and aprons. Before RNA extraction, virus is deactivated to prevent the possibility of generating aerosols during further downstream processing. Sample is heated at 56°C for 60 minutes in BSL-2 laboratory and then it is cooled down at room temperature.[14] Total viral RNA is extracted from the deactivated samples through company manufactured kits like Direct-zol™ RNA Prep, QIAamp™ Viral RNA Mini, FavorPrep™ Viral Nucleic Acid Extraction Kit or by manual extraction. All these kits contain common chemicals with different concentrations including lysis buffer, proteinase K, Trizol, and consumables i.e. spin columns. At first, sample is added in the lysis buffer solution that contains salts (Na^+ or K^+ or NH_4^+ salts), surfactants (Triton X-100 or SDS) and proteinase K and is incubated at 65^{0}C for 10 minutesfor the proper lysis of the cellular components. The surfactants (detergents) separate membrane proteins from biological membrane of the cell as their hydrophobic part surround the membrane and thus isolating the membrane proteins from membranes and make it vulnerable to Proteinase K. Proteinase K denatures capsid/ core proteins of virus freeing up the RNA; it also denatures RNases. Salts establish an ionic strength in the solution, which neutralize the charge on the nucleic acid backbone; this causes the RNA to become less hydrophilic. After incubation absolute ethanol (Ethanol or Isopropanol) is added to the lysate which removes the aqueous solvent shell around RNA molecules, allowing it to precipitate down by dehydrating the RNA. The total lysate is then dispensed into the filter paper/silica-based columns which attaches the RNA between the molecules of the column due to difference of chargers, as RNA carries negative charge due to its phosphate backbone while column molecules have positive charge. Columns are then washed with graded ethanol for the removal of contaminants including precipitated proteins and salts. RNA is then eluted in the nuclease free water and stored at -70^{0}C till RT-PCR processing.

RT-PCR Based Covid-19 Diagnosis

Real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) methodology is being used for the detection of causative viruses including COVID-

19 from respiratory secretions. RT-PCR assay, two step method, in which RNA is extracted from the sample and then complementary DNA (cDNA) is prepared. This cDNA is added to the master mix which contains the nuclease-free water, forward and reverse primer pair, a fluorophore-quencher probe, and a reaction mix (consisting of reverse transcriptase, polymerase, magnesium, nucleotides, and additives). The reaction mixture is run onto the real time thermocycler with the set PCR profile. Cleavage of the fluorophore – quencher probe during rRT-PCR generates a fluorescent signal which is detected by the thermocycler, and the amplification progress is recorded in real time. CDC recommended real time PCR employees one-step RT-PCR assay in which RNA is extracted and directly added to the master mix and skips the step of preparing cDNA from viral RNA. The cDNA preparation and amplification of targeted region takes place simultaneously in a single tube of PCR. The results are interpreted likewise the other two step rRT-PCR in which fluorescence of the cleaved fluorophore-quencher probe is generated and recorded in real time.[15] The schematic diagram for the real time is given in Figure.3.

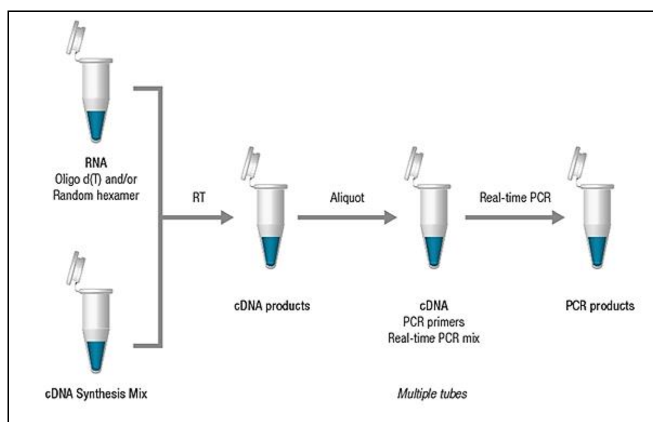

Fig.3: Schematic diagram for real time PCR.

Primers and probes specific to the targeted regions are designed. Primers pair (forward and reverse for both strands of targeted DNA) are the single stranded DNA molecule having 18 – 32 nucleotides targeting specific gene of our interest in viral genome. This is required to initiate the amplification of the targeted region. In contrast, probes are the short stranded, labelled DNA used to detect the presence or absence of the targeted nucleic acid in sampleby simply by hybridizing the targeted genome sequence. In RT-PCR, probe is attached to the downstream region of the specified primers so that the primers start synthesis and probe be cleaved by the polymerase and generate fluorescent signals. In the SARS-CoV-2 viral genomes, three regions having possible conserved sequences have been identified: (1) the RdRP gene (RNA-dependent RNA polymerase gene) in the first half of viral genome, (2) the E gene (envelope protein gene), and (3) the N gene (nucleo-capsid protein gene). Primers and probes specific for these regions are synthesized for the amplification of these regions and the detection of virus. Both the RdRP and E genes have high analytical sensitivity for detection, whereas the N gene provided poorer analytical sensitivity.[16]

At least two molecular targets should be included in the assay as the genome of the COVID-19 and other endemic coronaviruses are similar and there can be cross reactivity between these similar structures. This also helps in avoiding the potential genetic drift of SARS-CoV-2. Various investigators in different countries have used a number of these molecular targets for real-time including open reading frame 1b, nucleo-capsid gene, envelope and RNA-dependent RNA polymerase genes.[7,17] The CDC recommended covid-19 detection kits include three conserved nucleo-capsid genes regions of the virus and RNAse P gene of human.[18] The probe sequence used in routine is Black Hole Quencher-1 (BHQ1, quencher) and fluorescein amidite (FAM, fluorophore). Assay controls include a positive control which is plasmid based viral genome (mostly Cy5 labelled), a negative control i.e. saline and internal control which is human RNAse P (RNP) gene to assess the reliability of the results of the running rRT-PCR. The internal control, human RNP, is added in the kits to determine the presence of human genome in the sample along with the viral RNA. This validates the correct sampling procedure. All clinical samples should be tested for to assess specimen quality.[19]

More than seven types of SARS-CoV-2 nucleic acid diagnostic test kits have been developed and approved by WHO but the false negativity has continuously been reported by all the developed kits. The false negative rate (FNR) has been

explained by several factors in various reports which include the sampling procedures at inappropriate anatomical site, at different interval of infection which is affected by the different viral loads at various anatomical sites at different intervals of infection. Also, by the variations of viral RNA sequences at which the region-specific primer pair have been designed for amplification. This cause the primers not to bind the targeted region and ultimately no amplification of the viral region.[20]

Data Interpretation

Results are interpreted on the basis of Ct values at which detectable copy number of the virus are amplified. The Ct is the number of replication cycles required to produce a fluorescent signal (referring to the exponential amplification of genome), with lower Ct values representing higher viral RNA loads. A Ct value less than 40 is clinically reported as PCR positive as shown in figure 4. Quality controls run with each batch of the samples should have the performances as described below to ensure if the run of the batch has been successfully done:

- NTCs (No/negative template controls) may exhibit no fluorescence growth curves or Ct value more than 40 cycles that cross the threshold line.
- PTC (positive template controls) reaction produces a positive result with an expected Ct value for each target included in the test.
- Positive RP at or before 40 cycles for all clinical indicating the presence of sufficient nucleic acid from human RNase P gene and acceptable quality.

  The results are interpreted according to the exhibited performances of the quality controls and the interpretation is as follows:

- When all controls exhibit the expected performance, a specimen is considered negative or invalid if all 2019-nCoV markers (designated regions targeted by the kit being used) have Ct values more than 40 cycles including the RNAse P gene.
- When all controls exhibit the expected performance, a specimen is considered positive for 2019-nCoV if all markers (designated regions targeted by the kit being used) have Ct values less than 40 cycles including the RNAse P gene.

Also, number of positive designated regions targeted in the kit being used vary and are as per instructions of the kit e.g. three regions of nucleo-capsid gene (N1, N2, N3) are targeted by the CDC recommended kits in which sample is considered positive if any two of the designated regions have Ct values less than 40 including RNAse P gene.

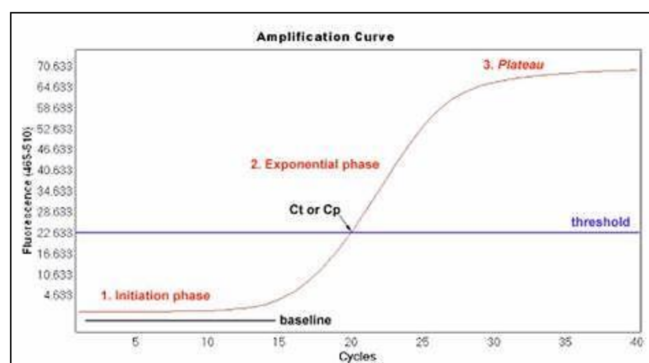

Fig.4: Real time PCR amplification curve and threshold cycle (Ct value).

Results may be inconclusive and invalid with respect to the expected performances of the quality controls. For the invalid results, residual RNA should be re-tested and if there is no residual RNA then re-extraction of the RNA from the remaining stored specimen is recommended.[21]

LIMITATIONS OF STUDY

There may be sampling issues that may cause the false negative results as if the swab does not touch the pharynx or is not fully soaked with the nasal fluid. Contamination of other residing microorganism may be the reason for the false positive results. Untrained supervision and staff may be a cause of false negative as well as false positive results. If anasymptomatic patient was infected with SARS-CoV-2 but has since recovered, PCR would not identify this prior infection, and control measures would not be enforced. In this case, immunological assays including patient's blood IgG levels should be measured in order to look for the past SARS CoV-19 infection.

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CONFLICT OF INETEREST

None to declare.

FINANCIAL DISCLOSURE

None to disclose.

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Author's Contribution

OS: Conception and acquisition of the published data and drafting the manuscript.

AS: Acquisition of published data and drafting the manuscript.

SM: Approval of final version to be published