Considerations in Real-time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for the Detection of SARS-CoV-2 from Nasopharyngeal Swabs

Priyadharshini Sekar¹, Godfred Antony Menezes²*, Pooja Shivappa³, Biji Thomas George⁴ and Ashfaque Hossain⁵

¹Sharjah Institute of Medical Research (SIMR), Research Institute of Medical Health Sciences, University of Sharjah, 27272, Sharjah, United Arab Emirate.
²Medical Microbiology and Immunology (MMI) Department, RAK College of Medical Sciences (RAKCOMS); Central Research Laboratory (CRL), RAK Medical and Health Sciences University (RAKMHSU); Clinical Microbiologist- RAK Hospital, 11172, Ras Al Khaimah, United Arab Emirate.
³Department of Basic Science; Central Research Laboratory (CRL), RAK Medical & Health Sciences University (RAKMHSU), 11172, Ras Al Khaimah, United Arab Emirate.
⁴Department of Surgery, RAK College of Medical Sciences (RAKCOMS); RAK Medical and Health Sciences University (RAKMHSU), 11172, Ras Al Khaimah, United Arab Emirate.
⁵Medical Microbiology and Immunology (MMI) Department, RAK College of Medical Sciences (RAKCOMS); Central Research Laboratory, RAK Medical and Health Sciences University (RAKMHSU), 11172, Ras Al Khaimah, United Arab Emirate.

Authors’ contributions

This work was carried out in collaboration among all authors. Authors PS, GAM, PS, BTJ and AH planned the need for this literature review. Authors PS, GAM and PS managed the literature searches and wrote the draft manuscript. Authors BTJ and AH edited the manuscript and finalized the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JPRI/2021/v33i1731309

Received 17 January 2021
Accepted 22 March 2021
Published 27 March 2021

*Corresponding author: E-mail: godfred@rakhsu.ac.ae;
ABSTRACT

Coronavirus Disease 2019 (COVID-19) was first reported in December 2019, in the City of Wuhan, China. Within the span of a few weeks, the disease had spread to other regions of China and eventually to different parts of the world. COVID 19 has affected 221 countries and territories around the world, with a total of 121,290,697 positive cases and 2,682,554 deaths as on March 17, 2021. Accurate disease diagnosis (for the SARS-CoV-2 virus and variants), coupled to patient isolation are currently critical strategies in restricting disease spread. Due to lack of time during this pandemic the diagnostics assays were not adequately validated. Infected individuals at times could potentially be missed by real-time reverse transcription polymerase chain reaction (rRT-PCR) for SARS-CoV-2 tests due to incorrect/inefficient sampling procedure, low limit of detection and epidemiology of the virus. rRT-PCR test results should be interpreted in conjunction with clinical examination and Computed Tomography (CT), particularly in suspected symptomatic individuals or those with epidemiological history of contact with known COVID-19 cases. Considering the above-mentioned constraints, the current scenario demands rapid and point-of-care tests for detection of SARS-CoV-2 in remote locations. To date, there is no reliable commercially available antigen detection kit. The infected subjects reveal low levels of antibodies against SARS-CoV-2 through the early period of infection. In addition, techniques such as, Digital RT-PCR technology and isothermal RNA amplification with electrochemical biosensors are some of the new technologies currently being developed to provide sensitive and specific SARS-CoV-2 antigen detection. The newly reported variant, SARS-CoV-2 VUI 202012/01 may not influence diagnostic outcomes as worldwide most PCR assays use two or more (including RdRp/ E/ N) reliable gene targets, besides S gene.

Keywords: COVID-19, SARS-CoV-2, real-time RT PCR, techniques, validation, results.

1. INTRODUCTION

Coronavirus Disease 2019 (COVID-19) was first reported in December 2019, in the city of Wuhan, Hubei Province of the People’s Republic of China. Within a span of a few weeks, the disease not only spread to other regions of China but also to other parts of the world. On 11 March 2020, WHO declared COVID-19 as a pandemic [1]. As of 17th March 2021, COVID-19 has affected 221 countries and territories around the world, with a total of 121,290,697 positive cases and 2,682,554 deaths reported across the world [2]. The causative agent for the Coronavirus Disease (COVID-19) is SARS-CoV-2, a novel virus belonging to the Coronaviridae family. The former name for Coronavirus Disease (COVID-19) was 2019-nCoV [3].

Nucleic acid amplification tests are the most common diagnostic tests for the detection of SARS-CoV-2. In addition to having an increased sensitivity and specificity, real-time reverse transcription polymerase chain reaction (rRT-PCR) possibly overcomes the many problems observed in molecular diagnosis of SARS-CoV-2. It includes detecting small number of viral RNA in order to reduce false negatives; differentiating between the different pathogens in order to reduce false positives; and carrying out multiple tests for a large inflow of samples [4,5].

This document focuses on the technicalities that are involved in the detection of SARS-CoV-2 from nasopharyngeal swabs, obtained from patients suspected of being affected by COVID-19, by rRT-PCR in a clinical laboratory.

2. SAMPLE COLLECTION

Safety precautions to be observed during sample collection, handling and processing of samples for the detection of SARS-CoV-2: Collection of nasopharyngeal swab is an invasive procedure. Knowledge of the anatomy of the upper respiratory tract is essential in order to collect this sample. Hence, only trained medical personnel should collect nasopharyngeal swabs. The medical personnel collecting the specimen should wear personnel protective equipment (PPE) which consists of a gown that covers arms, legs and has back closure, gloves, an N95 mask, a head cap, a face shield and shoe covers [6].

3. COLLECTION OF NASOPHARYNGEAL SWAB

The acceptable specimens for approved SARS-CoV-2 tests, depending on the assay are the following, a nasopharyngeal sample (NP); an oropharyngeal sample (OP); a nasal mid-
turbinateswab; an anterior nares (nasal swab) sample; nasopharyngealwash/aspirate or nasal wash/aspirate; a saliva sample (under supervision at home or testing center). Of the samples, nasopharyngeal, oropharyngeal, nasal mid-turbinate swab, and anterior nares (nasal swab) sample is to be collected by the collected by a trained medical personnel for appropriate sampling [7].

CDC recommends only the nasopharyngeal sample, while oropharyngeal sample is acceptable. In case both samples (NP and OP) are collected, they are aliquoted to increase the test sensitivity. For lower respiratory tract the samples collected are either of the following, bronchoalveolar lavage, tracheal aspirate, pleural fluid, lung biopsy or sputum. Other than sputum, the sample collection requires trained personnel; appropriate equipment and is performed for subjects with hospitalised with severe disease [7].

For NP swab collection, after asking the patient to remove the mask, ask them to blow their nose gently into a tissue to remove excess dirt from the nostrils. Ask the patient to tilt the head up. Insert the nasopharyngeal swab at an angle such that the thin flexible part of the swab can slide onto the floor of the nasal passage and reach the wall of the nasal cavity. Allow the swab to rest in that position for several seconds and gently pull out the swab while rotating it. Place the swab in the screw-capped plastic container that has the viral transport medium (VTM) [6]. Break off the stick of the swab, and screw the cap back in place tightly, to avoid any spillage. Clean the surface of the VTM tube with a surface disinfectant wipe, stick the label with the patient, and sample collection details. Place the sample in a double biohazard safety pouch that is lined with absorbent cotton, to aid in case the VTM leaks. Seal the pouch and place it in an upright position. The sample has to be maintained at 40°C. It can be stored at 40°C for up to 5 days and beyond that, it needs to be stored at -20°C to -80°C. Hence, samples are placed in an icebox with ice gel packs, and they are transported to the laboratory [6].

On arrival in the clinical laboratory, the sample should continue to be in the biohazard pouch until sample acceptance and entry of details in the laboratory accession area are performed. Following this, the samples should be removed from the biohazard pouch and placed in the rack in a biosafety cabinet or laminar airflow [6].

4. HANDLING OF SAMPLE SPILLAGE

There are instances when on arrival, samples might have spilled or leaked into the biohazard pouch. Ideally, another sample should be asked. However, in most cases this might be not possible. In such instances, the sample tube should be carefully removed from the biohazard pouch, the screw cap should be closed shut and the surface of the tube should be thoroughly wiped clean with 10% hypochlorite (concentration), followed by wiping off the tube with 70% alcohol (concentration). The biohazard pouch should be discarded in the red bin and the work surface should be immediately cleaned with 10% hypochlorite, followed by wiping with 70% alcohol. Non-propagative diagnostics (such as, nucleic acid amplification test [NAAT] & sequencing) is to be carried out in a Biosafety Level II (BSL-2) facility [4].

5. EXTRACTION OF RNA

The RNA extraction room should be under negative pressure [4]. The instruments present in the RNA extraction room are a bio-safety cabinet, RNA extraction machine, and computer. The sample IDs are first entered into the computer. To store this, a template of the RNA extraction plate may be made in a Microsoft Excel sheet and it may be labeled as plate set-up file Table 1. The sample IDs can be entered into the plate set-up file as shown in the table. Each RNA extraction plate should be labeled. For example P-1, P-2, P-3, and so on.

Example of a plate set-up file (Table 1). Plate -1 (P-1) indicates the number of the RNA extraction plate. Well indicates the wells into which the sample will be added. SL No. indicates the order of the samples, with sample ID that is added. This serial number can be marked on the sample tube and placed in a tube rack in the same order to enable easy addition of the sample to the extraction wells. Either the sample ID can be entered manually or if the ID number is barcoded, with a barcode scanner, it can be entered with the help of it. Once the sample IDs are entered into the system, the sample tubes with the respective serial numbers marked on them, must be correctly arranged in the tube rack, in the same order that it would be added to the RNA extraction plate.
Table 1. A template of the RNA extraction plate made in a Microsoft Excel sheet and labeled as plate set-up file

| Well | Sl.No. | Sample Id | Well | Sl.No. | Sample Id | Well | Sl.No. | Sample Id |
|------|--------|-----------|------|--------|-----------|------|--------|-----------|
| A    | 1      | 543590    | A    | 9      | 543598    | A    | 17     | 543604    |
| B    | 2      | 543591    | B    | 10     | 543599    | B    | 18     | 543605    |
| C    | 3      | 543592    | C    | 11     | 543600    | C    | 19     | 543606    |
| D    | 4      | 543593    | D    | 12     | 543601    | D    | 20     | 543607    |
| E    | 5      | 543594    | E    | 13     | 543602    | E    | 21     | 543608    |
| F    | 6      | 543595    | F    | 14     | 543603    | F    | 22     | 543609    |
| G    | 7      | 543596    | G    | 15     | 543604    | G    | 23     | 543610    |
| H    | 8      | 543597    | H    | 16     | 543605    | H    | 24     | 543611    |

6. PREPARATION OF THE RNA EXTRACTION MACHINE

The interiors and the probes of the RNA extraction machine should be cleaned as per the manufacturer’s recommendation. Ideally, the doors, handles, interiors, and the probes of the RNA extraction machine should be cleaned with 70% alcohol (concentration) and the UV light should be turned on for at least 15 minutes. This could be performed before starting any work in the extraction room [8].

7. PREPARATION OF THE BIOSAFETY CABINET II OR III

The work area of the biosafety cabinets should be cleaned with a 10% sodium hypochlorite solution, followed by wiping down with distilled water. This is done to prevent the corrosive action of sodium hypochlorite. Then the area is wiped with 70% ethanol, then allowed to dry for a minute following which the UV should be turned on for at least 15 minutes. In the case of biosafety cabinet III, the pass through box and the gloves attached to the glove box should also be cleaned in the same manner. Once the UV sterilization is done, raise the protective shield of the biosafety cabinet II to the recommended height and turn on the blower. Make sure the pressure inside the cabinet-level is at the recommended safety level. A height-adjustable chair should be used to carry on work in the biosafety cabinet. The height of the chair should be adjusted such that the face and the neck of the laboratory personnel should be above the opening of the cabinet [9].

8. PREPARATION OF THE RNA EXTRACTION PLATE

The RNA extraction plates would contain magnetic beads. Before taking the plate into the cabinet, place the extraction plate for few seconds on a magnetic plate. This will capture all the beads to the bottom of the wells of the plate. Before taking the extraction plate into the hood, mark the plate number on the side of the plate, so that the number is visible. The sealing foil/wrap of the extraction plate should be removed inside the cabinet [10].

9. WORKING IN THE BIOSAFETY CABINET II OR III

There must be a unidirectional workflow within the work area of the biosafety cabinet. The items that need to inside the cabinet are sample tubes that are placed in a rack, a vortex, RNA extraction plate, tip box, pipette stand, an empty rack to place the processed sample tubes, discarding containers for the tips, tissues and a 70% alcohol spray bottle. These items should be arranged in the same order in a unidirectional manner to avoid cross-contamination. Each item that is taken inside the cabinet should be thoroughly cleaned with 10% sodium hypochlorite, distilled water, and 70% ethanol if it is required [10]. Before beginning to add the samples to the wells in the extraction, ensure that the work materials are arranged in a unidirectional flow either from left to right or from right to left depending on the convenience of the personnel. However, when working in a biosafety cabinet III, the location of the sluice gate is to be kept in mind before deciding on the orientation of the direction of the workflow. Once everything in the biosafety cabinet is set up, the processing of samples for extraction of RNA can be initiated. Care should be taken to ensure that samples are added to the correct wells in the extraction plate, as predetermined in the plate set up. Since there are specific columns into which the samples need to be added, these columns can be highlighted with a marker pen, taking care to not touch the insides of the well.
Processing of the sample should only be performed by trained personnel who are protected in complete PPE equipment. Standard operation protocols must be adhered to when processing the samples. Briefly, the sample tube should be vortexed for 1-2 secs, the cap tapped (to make sure droplets adhering to the sides of the cap, drop down into the tube), and then it should be opened in such a way that the swab remains inside the tube. The cap of the sample tube may be held in the hand of the laboratory personnel, or, if working in a biosafety cabinet III, when it is difficult to hold the cap through the glove port, the cap may be placed on the work area over a thick wad of tissue that is pre-sprayed with 70% ethanol. The required volume of the sample is aspirated with the help of a pipette and dispensed into appropriate wells taking care to not touch the sides of the sample tube as well as the sides of the wells of the extraction plate. When dispensing the sample it should be done gently, such that aerosols are not generated. After the addition of the sample, the tip should be discarded in a discarding container that is lined with a double layer of autoclave quality, sealable, plastic bag. Close the sample tube, and place it on the empty rack, so that it serves as an indication that the sample in question has been processed.

If the VTM turns frothy on vortexing, then tilt the tube gently to help in aspirating the required volume of the sample. If the pipettes get in contact with this froth, then immediately, using a thick wad of tissue, wipe it down with 10% sodium hypochlorite, followed by distilled water, then 70% alcohol. Discard the tissue in the discard container. If during transfer of sample, a spillage occurs on the work area then immediately follow the same cleaning protocol - 10% sodium hypochlorite, followed by distilled water, then 70% alcohol [10].

Once all the samples are added to the RNA extraction plate, the plate has to be carefully taken out of the biosafety cabinet and placed inside the RNA extraction machine. The RNA extraction machine should be operated as per the manufacturer’s protocol. After the RNA extraction is completed, care should be taken to discard the probes/ tips in a biohazard pouch, sealed, and then placed in the discarding bin. After every round of extraction, it is desirable to clean the interiors of the extraction machine and turn on the UV for at least 10 minutes, before the next round of extraction of RNA begins. The extraction plates should be carefully taken out and placed in a biosafety cabinet for the transfer of RNA. RNA can be transferred into PCR strip tubes and placed at 40°C (for 1 hour) until used for setting up PCR, or it can be directly transferred into the PCR tubes (after addition of master mix) and the rRT-PCR can be set up immediately [10-12].

10. PRE-PCR AND SETTING UP THE rRT-PCR

Setting up the RT-PCR should happen in another separate clean room, which is also under negative pressure. The instruments present in the pre-PCR room are a PCR workstation, to set up the master mix and a biosafety cabinet II to add the RNA. Inside the PCR workstation should be a set of pipettes, a mini vortex, and a mini centrifuge. Inside biosafety cabinet II, should be a strip/plate centrifuge and a set of pipettes including a multi-channel pipette. Before beginning any work in the pre-PCR room, the work area of the PCR workstation as well as that of the biosafety cabinet II should be cleaned with 10% sodium hypochlorite, followed by wiping down with distilled water and then using 70% ethanol to finally clean everything. This includes cleaning of the vortex, mini centrifuge as well as the pipette sets. After this UV light needs to be turned on for at least 15 min [13].

The reagents for the real-time RT-PCR should be thawed on crushed ice. If not, it can be thawed in a coolant rack. Once the contents are thawed, gently vortex it for 1 sec and briefly spin it in a mini centrifuge. Tubes containing enzymes should never be vortexed because froth would be created. Setting up the PCR reaction should be according to the manufacturer’s protocol. The setting up of the PCR reaction should only be performed in the PCR workstation. The RNA should be added only in the biosafety cabinet II. When setting up a reaction, care should be taken to avoid the introduction of air bubbles. To avoid this, it is recommended to slightly keep the tip of the pipette at an angle and then dispense the contents of the tip onto the walls of the tubes. When aspirating contents from a tube, it is best to not touch the bottom surface of the tube. The addition of the extracted RNA should be performed in the biosafety cabinet II. After the addition of the RNA to the PCR strip tubes, it needs to be briefly mixed by spinning it for 1 minute in a plate or a strip centrifuge. As in the extraction room, the work in the RNA extraction
11. WORKING IN THE PCR ROOM

The PCR room also needs to be under negative pressure. This is the room where, the thermal cyclers are kept and operated. This room can also be used for the addition of RNA. Real-Time RT-PCR is set up in the thermal cycler as per the kit manufacturer’s protocol and standing operation protocols of the instrument. After completion of the PCR, the strip tubes or the plate should be carefully discarded in a biosafety hazard pouch and sealed, and then discarded in the biohazard discarding bin [15].

12. RESULT INTERPRETATION

Indications for performing rRT-PCR for COVID-19 include: confirming suspected cases; deciding on the release of confirmed patients from quarantine; screening asymptomatic individuals in close contact with confirmed case; and differential diagnosis of cases with unknown respiratory syndromes. rRT-PCR detects RNA in NP swab specimens in the first week, even before the onset of initial symptoms [16].

rRT-PCR kits detecting two or more genes are preferred to avoid false negatives. rRT-PCR diagnosis is made based on threshold cycle (Ct) value. Ct is the intersection between an amplification curve and a threshold line. It is a relative measure of the concentration of target in the PCR reaction. A lower Ct value denotes higher gene concentration in the sample. rRT-PCR is recommended (Gold Standard) for molecular testing. A major advantage of the test is amplification and analysis happening simultaneously. SARS-like bat coronaviruses, including both SARS-CoV and SARS-CoV-2 belong to the Subgenus Sarbecovirus. The molecular targets include structural genes such as, envelope glycoproteins Spike, S; Envelope, E; Transmembrane, M; Helicase, Hel; and Nucleocapsid, N. Species-specific accessory genes include RNA-Dependent RNA Polymerase, RdRp; Hemagglutinin Esterase, HE; Open Reading Frames, ORF1a and ORF1b. CDC recommends N1 and N2 targets. WHO recommends first-line screening with an E gene assay followed by a confirmatory test using the RdRp gene. Low Ct values indicating high viral loads may be used as an indication of transmissibility. Patients with high Ct values more than 35 can be considered non-infectious [17-26].

Though there are variations (no Standard Ct cut-off value) in interpretations according to diagnostic kits, generally Ct value of 35 or less is concluded as positive for COVID-19. The crucial factors for rRT-PCR based COVID-19 diagnosis to be considered include sample collection; sample transportation and storage; kit selection (pool of genes); processing of samples for RNA extraction and setting up PCR; Ct cut-off value and interpretations. A “positive” PCR result need not necessarily signify the presence of a viable virus. Viral RNA is, in some cases, detected even beyond week 6 by rRT-PCR [18-22,27]. Some centres have tried pooled samples (up to 30 samples) for to reduce the cost of testing for SARS-CoV-2 in asymptomatic people. Pooled samples should be tested using reliable kits and should be interpreted cautiously. It could be useful in scenarios like returning groups of workers to a workplace. Lower disease prevalence may enable a laboratory to use a larger optimal pool size. As the prevalence of COVID-19 increases, the cost savings of a pooling strategy decreases because more pooled tests will return positive results and those specimens will need to be retested individually. Negative results from pooled testing should not be treated as definitive. If a patient’s clinical signs and symptoms are inconsistent with patient management, then the patient should be considered for individual testing [7,19].

13. DISCUSSION

Among the target genes for COVID-19, RdRp has been found to be most sensitive. Tests with two probes detects bat-related SARS coronaviruses and other coronaviruses (Pangolins and specifically SARS-CoV-2 (RdRp-p2). The RdRp/Hel rRT-PCR developed was more sensitive than RdRp-p2 method [23]. As rRT-PCR requires sophisticated laboratory and specific equipment with trained personnel, it limits the use for rapid/point of care diagnosis [24,28]. It is vital to have more of affordable and valid point of care diagnostic tests during this current pandemic [28].

The genome of SARS-COV-2 (beta corona viruses) is structured in the order of 5′-replicase (ORF1a/b); spike (S); envelope (E); membrane
The preferred targets of rRT-PCR are S and N genes (structural; abundantly expressed/conserved) and RdRp and replicase open reading frame (ORF) 1a/b genes (non-structural) [30]. For COVID-19, the protocols of a number of RT-PCR assays used by different institutes have recently been made available online [31]. These assays target the ORF1a/b, ORF1b-nsp14, RdRp, S, E, or N gene of SARS-CoV-2, and some are nonspecific assays that would detect SARS-CoV-2 and other related betacoronaviruses such as SARS-CoV [24,32]. The currently available RNA diagnostic assays for SARS-CoV-2 use two or more target genes with different conclusion conditions. In a single test, it requires two target genes for the test to be positive (where RdRp must be positive). During inconclusive results, on repeat if RdRp alone is positive in the retest, the result can also be considered positive. Further, if RdRp is negative (though both N and E are positive) the result is not concluded as positive. As the sensitivity of the rRT-PCR is not absolute (around 80%), the results should be interpreted considering other indications [25].

False negative COVID-19 results: The reasons for false negatives are, a. improper nasopharyngeal swab collection; b. errors in sample preparation; c. equipment or laboratory personnel error; d. reporting errors; e. sample labeling errors; f. the timing of the test (very early or too late) relative to the course of the infection. It is advisable to report negative result as “no virus detected” in place of “not infected with the virus”. False negative results are more concerning than false positive results, because infected persons (asymptomatic) may infect others, as they are not subjected to quarantine [33].

The results of rRT-PCR results for SARS-CoV-2 needs to be interpreted cautiously (Table 2). In case of negative rRT-PCR (SARS COV-2) results in subject with clinical suspicion of COVID-19, multiple sample types including lower respiratory tract sample should be tested. Computed tomography (CT) images in conjunction with rRT-PCR results facilitates disease management. Appropriate sampling procedures, good laboratory practices, and using superior-quality extraction and rRT-PCR assays advance the method and decrease inaccurate results [17]. Considering the fact that SARS-CoV-2 is undergoing continued genetic variability, two or more reliable gene targets can alleviate the risk of loss of sensitivity or specificity [34].

+ = Ct value ≤35 (manufacturer’s kit protocol to be referred to). Invalid test results: if internal controls or positive controls do not amplify or if the negative control amplifies. If an invalid test result is obtained, the sample needs to be re-tested either by extracting fresh RNA from the existing sample or by collecting a new sample from the patient.

| COVID-19- E | COVID-19-RdRp/Hei | COVID-19- N | Interpretation |
|-------------|-------------------|-------------|----------------|
| +           | -                 | -           | Presumptive positive* |
| -           | +                 | -           | SARS-CoV-2 RNA is Detected** |
| -           | +                 | +           | SARS-CoV-2 RNA is Detected** |
| -           | -                 | +           | SARS-CoV-2 RNA is Detected** |
| +           | -                 | -           | SARS-CoV-2 RNA is Not Detected |
| +           | +                 | +           | SARS-CoV-2 RNA is Detected |
| +           | +                 | -           | SARS-CoV-2 RNA is Detected** |

Table 2. Finer aspects in interpreting results of real-time reverse transcription polymerase chain reaction (rRT-PCR) for the detection of SARS-CoV2
*Sarbecovirus RNA is detected, whereas SARS-CoV presumptive positive. Repeat assay with the extracted RNA stored (-80°C) specimen aliquot, where RdRp must be positive for the test to be positive.

** rRT-PCR for SARS-COV2 relies on presence of two or more target genes in the patients sample. The Ct values of gene targets depends upon the viral load of the patient. The presence of one reliable gene target within the defined Ct range for SARS-CoV-2 is all that is required for a sample to be positive. If one gene target is detected at very low Ct values (high measurable range) and the other targets in the assay are not detected, the sample needs to be re-tested. If inconclusive results are either obtained a second time, the sample needs to be re-tested by re-extracting fresh RNA from the existing sample or by collecting a new sample from the patient and testing the new sample. Negative target (RdRp/He or N genes) results are suggestive of specimen at concentrations below the limit of detection; a mutation in the corresponding target region, or other factors. Repeat assay with the extracted RNA stored (-80°C) specimen aliquot, where RdRp must be positive for the test to be positive.

The E gene assay is used as the first line screening tool, then followed by confirmatory testing with an RdRp gene assay. The N gene assay can eventually be analyzed as an additional confirmatory assay [24]. Lower Ct values usually are associated with severe outcomes. Ct values may be advantageous in foreseeing the clinical course and prognosis of patients with COVID-19 [35]. The viral load during the second infection could be higher with detectable antibody title suggestive of reinfection [36]. rRT-PCR (SARS-CoV-2) results for N and Orf 1b genes in an asymptomatic patient was found to be with Ct values of 30-32 and higher viral load (inversely related to Ct value) were detected soon after the onset of symptoms, with higher viral loads detected in the nose [37]. rRT-PCR for SARS-CoV-2 test results should be interpreted in conjunction with clinical examination and Ct, particularly in suspected symptomatic individuals or those with epidemiological history [38]. Further the current scenario calls for developing rapid and point-of-care tests for detection of SARS-CoV-2 in remote locations [39]. To date, there is no reliable commercially available antigen or antibody test for detection of SARS-CoV-2 infection. The isothermal RNA amplification with electrochemical biosensors is the need of the hour [39,40]. As SARS-CoV-2 is a zoonotic virus, besides humans it is important to monitor the transmission of SARS-CoV-2 infection among pet and domestic animals [41]. Lately the authorities of the United Kingdom of Great Britain and Northern Ireland have reported SARS-CoV-2 VUI 202012/01(new SARS-CoV-2, variant 01 in Dec 2020), WHO, 2020 [42]. This variant is thought to spread more rapidly and there are ongoing investigations to detect if this variant is connected to alterations in the severity of the disease, antibody response, vaccine efficiency or diagnostic PCR assays outcome. The PCR assays using only S gene target was found to be affected due to deletion at position 69/70. However, worldwide most PCR assays use two or more reliable gene targets. Thus, the variants may not have influence on the diagnostics outcome to a major extent. Research is underway to infer if this variant is related to deviation in severity of clinical presentation, antibody response or vaccine efficacy [42].

14. CONCLUSION

Even though the available modern molecular diagnostic assays do not take care of point of care diagnosis, they offer a platform for processing a large number of samples. Initially, up to 80% of the COVID-19 positive cases went undetected leading to transmission of infections. An accurate diagnosis is critical in restricting its spread. Due to lack of time during this pandemic the diagnostics assays were optimized or validated with inadequate clinical sample size. The infected individuals, at times, may be missed by rRT-PCR (SARS-CoV-2) tests due to improper diagnostic timing, low sensitivity of the test, low viral load and novelty of the disease. rRT-PCR (SARS-CoV-2) test results should be interpreted in conjunction with clinical examination and Ct, particularly in suspected symptomatic individuals or those with epidemiological history. Considering the constraints in rRT-PCR (SARS-CoV-2), there is dire need for continuous optimization and progressing at Digital RT-PCR technology. Further, the current scenario calls for developing rapid and point-of-care tests for detection of SARS-CoV-2 in remote locations. There are quite a few rapid, point of care tests, such as Lumex Instruments' Microchip RT-PCR COVID-19 Detection System and Abbott’s famous ID NOWTM Instrument and ID NOWTM COVID-19 Test Kit are in development. To date, there is no reliable commercially available antigen (due to
low levels) or virus detection kit. Further, subjects reveal low levels of antibodies against SARS-CoV-2 through the early period of infection. In addition, isothermal RNA amplification with electrochemical biosensors is the need of the hour. As SARS-CoV-2 is a zoonotic virus, it is important to monitor the transmission of SARS-CoV-2 infection among pets and domestic animals. The authorities in United Kingdom of Great Britain and Northern Ireland have recently reported SARS-CoV-2 Variant (SARS-CoV-2 VUI 202012/01). It is thought to spread more rapidly and there are ongoing investigations to detect if this can influence diagnostic PCR assays. However, worldwide most PCR assays use two or more (including RdRp/ E/ N) reliable gene targets, besides S gene. Thus, the variants may not have influence diagnostics outcome to a major extent. Research is underway to infer if this variant is related to deviation in severity of clinical presentation, antibody response or vaccine efficacy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. WHO. Novel Corona virus (2019-nCoV) situation report – 1; 2020. Available:https://www.who.int/docs/default-source/coronaviruse/situation-reports/20200121-sitrep-1-2019-ncov.pdf. Accessed: 27 Dec 2020.
2. Worldometer. COVID-19 corona virus pandemic; 2021. Available:https://www.worldometers.info/coronavirus/ Accessed: 17 Mar 2021.
3. Ludwig S, Zarbock A. Coronaviruses and SARS-CoV-2: A Brief overview. Anesthesia & Analgesia. 2020;131(1):93-96. DOI: 10.1213/ANE.0000000000004845
4. WHO. Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases: interim guidance. World Health Organization; 2020. Available:https://apps.who.int/iris/handle/10665/331329. [Accessed: 27 Dec 2020].
5. Axell-House DB, Lavingia R, Rafferty M, Clark E, Amirian ES, Chiao EY. The estimation of diagnostic accuracy of tests for COVID-19: A scoping review. Journal of Infection. 2020;81(5):681-697.
6. Qian Y, Zeng T, Wang H, Wang H, Xu M, Chen J, et al. Safety management of nasopharyngeal specimen collection from suspected cases of corona virus disease 2019. Int J Nurs Sci. 2020;7(2):153-156.
7. CDC. Centers for disease control and prevention: Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens for COVID-19; 2020. Available:https://www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html. [Accessed 27 Dec 2020].
8. Al-Saud H, Al-Romaik K, Bakheet R, Mahmou L, Al-Harbi N, Alshareef I, et al. Automated SARS-COV-2 RNA extraction from patient nasopharyngeal samples using a modified DNA extraction kit for high throughput testing. Ann Saudi Med 2020;40(5):373-381.
9. Mourya DT, Sapkal G, Yadav PD, Belani M, Shete SK, Gupta N. Biorisk assessment for infrastructure & biosafety requirements for the laboratories providing coronavirus SARS-CoV-2/(COVID-19) diagnosis. Indian J Med Res. 2020;151(2&3):172–176.
10. Klein S, Müller TG, Khalid D, Sonntag-Buck V, Heuser AM, Glass B, et al. SARS-CoV-2 RNA extraction using magnetic beads for rapid large-scale testing by RT-qPCR and RT-LAMP. Viruses 2020;7:12(8):863. DOI: 10.3390/v12080863.
11. Tripathi M, Kumar S. Epidemiology, treatment and microbiological surveillance of SARS-CoV-2. Annu Res Rev Biol. 2020;35(5):114-121.
12. Bhatia R, Chaudhary R, Khurana SK, Tiwari R, Dhama K, Gupta VK, Singh RK, Natesan S. Strengthening of molecular diagnosis of SARS-CoV-2 / COVID-19 with a Special Focus on India. J Pure Appl Microbiol. 2020;14(suppl 1):789-798.
13. Mifflin TE. Setting up a PCR laboratory. CSH protocols; 2007. Available:https://doi.org/10.1101/pdb.top1.
14. Lin C, Ye R, Xia YL. A meta-analysis to evaluate the effectiveness of real-time
PCR for diagnosing novel coronavirus infections. Genet Mol Res: GMR. 2015;14(4):15634–15641.

15. Santarpia JL, Rivera DN, Herrera VL, Morwitzer MJ, Creager HM, Santarpia GW, et al. Aerosol and surface contamination of SARS-CoV-2 observed in quarantine and isolation care. Sci Rep. 2020;10(1):12732.

16. Cheng MP, Papenburg J, Desjardins M, Kanjilal S, Quach C, Libman M, et al. Diagnostic Testing for Severe Acute Respiratory Syndrome-Related Coronavirus 2: A Narrative Review. Ann Intern Med. 2020;172(11):726–734.

17. Tahamtan A, Ardebili A. Real-time RT-PCR in COVID-19 detection: Issues affecting the results. Expert Rev Mol Diagn. 2020;20(5):453–454.

18. Sethuraman N, Jeremiah SS, Ryo A. Interpreting diagnostic tests for SARS-CoV-2. JAMA. 2020;323(22):2249–2251.

19. Lohse S, Pfuhl T, Berkó L, Gärtner B, Raza M, et al. Pooling of samples for testing for SARS-CoV-2 in asymptomatic people. Lancet Infect Dis 2020;20(11):1231–1232.

20. Afzal A. Molecular diagnostic technologies for COVID-19: Limitations and challenges. J Adv Res. 2020;26:149-159. DOI: 10.1016/j.jare.2020.08.002. PMID: 32837738; PMCID: PMC7406419.

21. Islam KU, Iqbal J. An update on molecular diagnostics for COVID-19. Front Cell Infect Microbiol. 2020;10:560616. Published: 2020 Nov 10. DOI: 10.3389/fcimb.2020.560616.

22. Okamoto K, Shirato K, Nao N, Saito S, Kageyama T, Hasegawa H, et al. Assessment of real-time RT-PCR kits for SARS-CoV-2 detection. Jpn J Infect Dis. 2020;24:73(5):366-368. DOI: 10.7883/yoken.JJID.2020.108. Epub: 2020 Apr 30. PMID: 32350226.

23. Chan JF, Yap CC, To KK, Tang TH, Wong SC, Leung KH, et al. Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/Hel real-time reverse transcription-pcr assay validated in vitro and with clinical specimens. J Clin Microbiol 2020;23:58(5):e00310-20. DOI: 10.1128/JCM.00310-20. PMID: 32132196; PMCID: PMC7180250.

24. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020;25(3):2000045. DOI: 10.2807/1560-7917. ES.2020. 25.3.2000045. Erratum in: Euro Surveill. 2020 Apr;25(14): Euro Surveill. 2020 Jul;25(30) PMID: 31992387; PMCID: PMC6988269.

25. Wu Y, Xu W, Zhu Z, Xia X. Laboratory verification of an RT-PCR assay for SARS-CoV-2. J Clin Lab Anal. 2020;34:e23507. DOI: https://doi.org/10.1002/jcla.23507.

26. Colton H, Ankcorn M, Yavuz M, Tovey L, Cope A, Raza M, et al. Improved sensitivity using a dual target, E and RdRp assay for the diagnosis of SARS-CoV-2 infection: Experience at a large NHS Foundation Trust in the UK. The Journal of infection. 2020;S0163-4453(20)30339-X. Available:https://doi.org/10.1016/j.jinf.2020.05.061.

27. Chang MC, Hur J, Park D. Interpreting the COVID-19 test results: A guide for physiatrists. Am J Phys Med Rehabil. 2020;99(7):583–585. DOI:https://doi.org/10.1097/PHM.00000000000001471.

28. Notomi T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000:28(12):636–663.

29. Chan JF, Kok KH, Chu Z, Chu H, To KK, Yuen S, Yuen KY. Genomic characterization of the 2019 novel human-pathogenic coronavirus isolated from a patient with atypical pneumonia after visiting Wuhan. Emerg Microbes Infect. 2020;9:221–236. DOI: 10.1080/22221751.2020.1719902.

30. Chan JF, Lau SK, To KK, Cheng VC, Woo PC, Yuen KY. Middle East respiratory syndrome corona virus: Another zoonotic betacoronavirus causing SARS-like disease. Clin Microbiol Rev. 2015;28:465–522. DOI: 10.1128/CMR.00102-14.

31. WHO. All technical guidance on COVID-19 - select topic from drop down menu; 2020. Available:https://www.who.int/emergencies/diseases/novel-coronavirus-2019/technical-guidance-publications. Accessed: 27 Dec. 2020.

32. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, Ng DYM, et al. Molecular diagnosis of a novel coronavirus (2019-nCoV) causing an outbreak of
pneumonia. Clin Chem. 2020;66(4):549-555.
DOI: 10.1093/clinchem/hvaa029.
33. Stites EC, Wilen CB. The Interpretation of SARS-CoV-2 Diagnostic Tests. Med (N Y) 2020;1(1):78-89.
DOI: 10.1016/j.medj.2020.08.001
34. Peñarrubia L, Ruiz M, Porco R, Rao SN, Juanola-Falgarona M, Manissero D, ET AL. Multiple assays in a real-time RT-PCR SARS-CoV-2 panel can mitigate the risk of loss of sensitivity by new genomic variants during the COVID-19 outbreak. Int J Infect Dis. 2020;97:225-229.
DOI: 10.1016/j.ijid.2020.06.027. Epub: 2020 Jun 12.
PMID: 32535302; PMCID: PMC7289722.
35. Rao SN, Manissero D, Steele VR, Pareja J. Correction to: A systematic review of the clinical utility of cycle threshold values in the context of COVID-19. Infectious Diseases and Therapy. 2020;9(3):587.
DOI: 10.1007/s40121-020-00328-z.
36. Alonso F de OM, Sabino BD, Guimarães MAAM, Varella RB. Recurrence of SARS-CoV-2 infection with a more severe case after mild COVID-19, reversion of RT-qPCR for positive and late antibody response: Case report. J Med Virol 2020;14:10.1002/jmv.26432.
DOI: 10.1002/jmv.26432.
37. Zou L, Ruan F, Huang M, Liang L, Huang H, et al. SARS-CoV-2 viral load in upper respiratory specimens of infected patients. N Engl J Med. 2020;19;382(12):1177-1179.
DOI: 10.1056/NEJMc2001737. Epub: 2020 Feb 19.
38. Li Y, Yao L, Li J, Chen L, Song Y, Cai Z. Stability issues of RT-PCR testing of SARS-CoV-2 for hospitalized patients clinically diagnosed with COVID-19. J Med Virol. 2020;92(7):903-908.
DOI: 10.1002/jmv.25786.
39. Microchip RT-PCR COVID-19 (SARS-CoV-2) detection system. Lumex Instrum - Lab Anal Equip Manuf; 2020. Available:https://www.lumexinstruments.com/applications/covid-19_detection_system.php Accessed: June 13, 2020.
40. Ji T, Liu Z, Wang G, Guo X, Akbar Khan S, Lai C, et al. Detection of COVID-19: A review of the current literature and future perspectives. Biosens Bioelectron. 2020;166:112455.
DOI: 10.1016/j.bios.2020.112455. Epub: 2020 Jul 21.
PMID: 32739797; PMCID: PMC7371595.
41. Li C, Ren L. Recent progress on the diagnosis of 2019 Novel Coronavirus. Transbound Emerg Dis. 2020;67(4):1485-1491.
DOI: 10.1111/tbed.13620. Epub: 2020 May 31.
PMID: 32395897; PMCID: PMC7272792.
42. WHO. SARS-CoV-2 variant – United Kingdom of Great Britain and Northern Ireland. Disease Outbreak News; 2020. Available:https://www.who.int/csr/don/21-december-2020-sars-cov2-variant-united-kingdom/en/ Accessed: 27 Dec 2020.

© 2021 Sekar et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.