

A Review of Challenges in Performing RT- PCR for Covid-19 in Rural Healthcare Settings.

Abstract:

COVID-19 pandemic has thoroughly shaken the community globally including remote rural areas of all countries. Testing for SARS Cov-2 and clinical laboratory became the priority in hospital and healthcare settings during this pandemic. WHO confirmed about the specificity and sensitivity of RT-PCR and announced the RT-PCR as the confirmatory test for the Covid-19 investigation. Governments mobilized all **its** key resources towards establishing laboratories and enhance the number of diagnostic tests to curb the spread of pandemic. In rural and resource limited settings of India, molecular technique like RT-PCR faced **several** problems like improper laboratory set up, deficiency of trained health professionals and many setbacks. This review reflects on challenges experienced in rural and resource limited settings during Covid-19 pandemic.

Keywords: COVID-19, SARS Cov-2, Molecular diagnosis, Rural **and** Resource Limited Setting, RT-PCR

Introduction:

PCR is a technique that functions with either Deoxyribose nucleic acid (the genome) or ribonuclease acid (the transcriptome) of any living creature. The genome can generate data that are instructive and informative for future studies. (1) Every cell consists of the same DNA structure, the same mutations, and polymorphism. The transcriptome is an mRNA complement wherein changes happen with the physiology, pathology, or development. (1, 2) This makes the data inside the transcriptome inherently versatile and variable. If this uncertainty is combined with the technological limitations of any reverse transcription (RT)-PCR assay, the achievement of a technically accurate and biological result becomes difficult. (1)

Some technical aspects like template quality, operator variability, the RT process itself, and subjectivity in data analysis and reporting make it difficult the interpretation data related to real-time RT-PCR. (1,2) RT PCR takes just a few hours, compared to conventional methods of DNA cloning and replication, which mostly take days. PCR is extremely sensitive and needs the least template for precise sequence detection and amplification. (1, 3)

In resource-limited environments, major obstacles include high costs, technological difficulty sample transport from the periphery, and quality control. Due to a lack of resources and facilities, sensitive laboratory processes like RT PCR cannot be performed in

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an unsophisticated manner. (2) In the future, a generalization of testing coupled with price reduction tactics that will encourage healthy competition between various companies will allow viral load testing to be carried out in resource-poor environments. We will discuss the difficulties in processing RT PCR in rural or resource-limited areas.

Methodology:

The searching was done with the help of online databases (MEDLINE, EMBASE, PubMed, Google scholar). The online database search was conducted using the following Medical subject heading terms and keyword combinations: ("RT PCR"[MeSH Terms] OR "RT PCR "[All Fields]) AND ("Challenges"[Subheading] OR ("Challenges for diagnosis"[All Fields] AND "difficulties"[All Fields]) OR "rural and resource-limited setting"[All Fields] OR "rural and resource-limited setting and challenges"[MeSH Terms]). Manual searching was also done. The selection of the articles was based on the abstracts and titles. The journal, authors, or institution of the publications were not deprived blindly by the reviewers. Two reviewers checked and scrutinized all applicable references.

Data extraction:

Two analysts performed data extraction using a full manuscript available from the authors. Inclusion of data was with RT PCR/method, RT PCR/challenges, RT PCR/primer and probe, RT PCR/RT PCR kit, RT PCR/sampling procedures, RT PCR/detection of viral RNA, RT PCR/assay, RT PCR/result, RT PCR/diagnostic use, RT PCR/limitations, RT PCR/Human resource issue, RT PCR/logistics issue, RT PCR/Enzyme.

Inclusion criteria:

This review study included review articles, systematic reviews, conference proceedings, research articles, editorials.

Exclusion criteria:

The studies eliminated the manuscript published in non-indexed journals and any language other than English.

Detection of Viral RNA:

At the time of the initial outbreak, the researcher found the causative agent of Covid-19 and it was named SARS Cov-2. Extraction of total RNA was done from the bronchoalveolar lavage fluid sample. RNA was reverse transcribed into multiple copies of DNA and amplification of RNA was done with the help of RT PCR. (4) The quantity of RNA was decreased during this process. With a process of sequencing by synthesis, the amplified DNA was sequenced. The complete SARS-CoV-2 genome was obtained by overlapping DNA regions that were also high-abundance sequences (5,6).

At the initial stage of the Covid-19 pandemic, identification of speculative cases was done by using RT PCR assays and assured with genome sequencing. Because genome sequencing is more costly and laborious than RT-PCR. Although RTPCR is a successful technique, challenges seem in the overall process of detecting the viral RNA (4-6).

Sampling procedures:

They used eight types of samples for RT PCR detection of SARS-CoV-2 as follows: bronchoalveolar lavage fluid, fibrobronchoscopy brush biopsy, sputum, nasal swabs, pharyngeal swabs, stool, blood, and urine (5). The higher viral load or positive rate were observed with bronchoalveolar lavage fluid, followed by sputum, nasal swab, pharyngeal swabs respectively. All these samples have been recommended by the World Health Organization for the detection in mobile patients (4,5). The positive rate for SARS-CoV-2 has been seen at the peak level in upper respiratory samples instantly after symptom onset and reduced with time after 1 week (5,6).

Typically, nasopharyngeal swab samples are obtained because this collection method is comparatively less intrusive. Lower respiratory specimens include sputum, endotracheal aspirate, and bronchoalveolar lavage (3,5). If the productive cough is present in patients, then sputum can be produced. Sputum production is not possible in asymptomatic or presymptomatic patients. It is a big challenge for collecting lower respiratory samples due to the risk of exposure to SARS-CoV-2 for personnel collecting specimens (5). There is a risk due to close contact with severe COVID-19 patients and irritation of respiratory airways during sampling and causes sneezing (5,6).

The sample receiving time should be the shortest to reach up to the laboratory after collection. Sample from the patient is stored at 2-8°C for up to 72 hours after collection. If there is the expectation of delay in testing or shipping, specimens are stored at -70°C or below (2,3).

For nasopharyngeal or oropharyngeal washes, sputum, endotracheal aspirate, and bronchoalveolar lavage, the maximum storage period is 2 days, and for nasopharyngeal and oropharyngeal swabs, serum, and whole blood, 5 days (4).

Sterile transport tube contains 2-3 ml of any medium and swab should be placed instantly into the medium tube. The medium should be used by anyone from viral transport medium (VTM), amies transport medium, phosphate-buffered saline, sterile saline. This test is designed for analysis of the sample with a specific medium (5,6).

The WHO recommends dacron or polyester flocks' swabs during the sample collection. Calcium alginate swabs or swabs with wooden shafts are to be avoided because it can inactivate some viruses and may inhibit molecular procedures. (5,6) The swab should reach a depth equal to the distance from nostrils to the outer opening of the ear, indicating contact with the nasopharynx. Labelling must be proper for each specimen container with the patient's ID number, the date of the sample collection, unique laboratory requisition number, specimen type (6).

Release of viral RNA:

The RNA is covered within nucleocapsid virions. The extraction of high-quality RNA is the first step in the detection of SARS Cov-2 in patient samples. RNA gets degraded easily by Ribonucleases. Incorrect extraction of RNA results in viral RNA loss or degradation. The RNA extraction process is time-consuming and needs laboratory equipment and trained technician (5,6).

The presence of residual salts and organic solvents spoils the quality of RNA, whereas divalent cations can inhibit RT-PCR. The growing demand for RNA extraction kits burdens the global distribution networks and suppliers which hampers the ability to rapidly diagnose RNA extraction kits for COVID-19.(6)

Viral RNA is difficult to access as it is packed by a viral protein coat. The detergent used for breaking viral coat can also prove a hindrance during PCR. (6)

RT PCR Assays:

SARS-CoV-2 detection targets include the N, E, and S protein-encoding genes, the open reading frame 1ab (Orf1ab), and the RNA-dependent RNA polymerase (RdRP) gene. Analytical particularity of RT PCR assays is affected by the choice of target. (6)In point of view, an outbreak of Covid-19, Seven RT-PCR assays produced by scientists around the globe for the diagnosis of COVID-19 assays were quickly made available by the WHO.(6)

Several limitations and issues arose during RT PCR assays and the use of a protocol for the first time. The positive detection rate was low and suggests a high rate of false negatives. These false-negative results were arisen due to errors in sampling and wrong selection of the patient sample, viral load variations, procedure difference, and the limitations of detection for various RTPCR kits. (6)

A variety of RT-PCR assays were developed for different RNA or DNA including a commercial ready-to-use RT-PCR kit (Artus Biotech, Hamberg, Germany). A process like gel electrophoresis was time-consuming and liable to false-positive results from amplicon contamination and though early assays are based on traditional designs that required a post-amplification product process. Detection and quantification of a fluorescent signal generated during amplification are basic for RT PCR assays. A fluorescent signal does not need post-amplification processing and therefore one can avoid the risk of template contamination. (7)

DNA is as tough as old boots, but RNA is extremely intricate once removed from its cellular habitat. Therefore, the RNA purification process is much troublesome than that of DNA.

An RNA template required for an RT-PCR assay must accomplish the following criteria:

1. If quantitative results are required, RNA must be of the highest quality.
2. In the case of the intron (small gene), the RNA template should be free of DNA
3. There must be no co-purification of inhibitors of the RT-step.
4. RNA must be nuclease-free for a long storage period. (3)

Challenges while performing RT PCR:

Master Mix Preparation:

Nowadays prepared master mix is available commercially in the market, but if unavailable then needs to be prepared very carefully. An excess amount of dNTPs can show the

inhibitory effect on PCR. An increase in the concentration of MgCl₂ will suddenly produce smearing or extraneous bands and a decrease in concentration will prevent getting bands. The high amount of template DNA inhibits the process of PCR. (6,7)

In the reagent preparation room, positive pressure has to be maintained to lessen the contamination. The laboratory is designed for the preparation and storage of PCR reagents including master mix. Assurance of this type of maintenance is not possible in a resource-limited setting (7)

Primer design:

Primer concentration is responsible for the complete absence or presence of bands. It is a crucial step for any known sample using PCR to target and amplify a known nucleotide sequence of interest. Appropriately designed primers increase the amplification of PCR effectively and also the extraction of the target sequence with great specificity. (8)

The distinction between the amplified product from cDNA and an amplified product derived from contaminating genomic DNA allows the primer design. (2,3)

A false-positive result mostly because of sample contamination can occur mainly due to one or more of the primer and probe NTC reactions. (2) Annealing temperature and time, and the number of accessible templates affect the probability of primer annealing if primers are excessive.

For primer design, computer and internet facilities with electricity must be present in the rural testing area. But the scarcity of electricity and internet are common in villages of India.

Enzymes:

Storage purpose is needed for enzymes to be working at their optimum temperatures. A lyophilized form of the enzyme can also work out in such a condition. Prevention of enzymes from freezing completely is done at glycerol storage at -20° C. Enzymes should be diluted for utilization in RT PCR test with molecular grade water or **DNase & RNase free water**. (2,3)

Social and environmental setting:

The approach for diagnosis can be difficult because more than 60% population of India lives in a rural area. Location and environmental factors affect the diagnosis. Screening, diagnosis, and treatment become more problematic due to social disgrace and fear of isolation and prejudice in villages which can lead to a traumatic effect. (3)

Human resource issue:

The primary challenge for the search of local laboratory technician expert with molecular biology in a rural setting. There is a lack of troubleshooting and maintenance in the countryside. (1) Health care providers worldwide in rural areas also encounter a range of difficulties and obstacles when attempting to deliver services. (1,2)

The number of highly trained health professionals is low at all levels due to the cost of training and remuneration. A broader spectrum of the disease requires a rise in general investment in healthcare systems, infrastructure, and human resources. Trained health professionals refuse to give their service in rural areas due to lack of well-equipped labs and

improper organization. (3) During the Covid-19 pandemic, laboratory staff has worked more than 8 hours due to a large amount of sample in India. There should be a reduction in time for staff. 10 hours rest is an important key factor in the resistance of Covid-19.

Factors like handling of samples and treatment can be responsible for false-negative results. There are lots of reasons for the failure of the RT PCR test or false-negative results.(6,7) These may include an inappropriate collection of samples, loss or deterioration of the target RNA during transport and storage of the sample, ineffective extraction of RNA from the specimens (e.g., nasal swabs), poor RNA purification, and ineffective removal of sample impurities. Collection, storage, handling, and treatment of samples are critical for accurate and meaningful diagnosis. (7) False-negative results may appear for RT PCR due to amplification inhibitors present in the sample or less load of the organism in the sample. This happens with inappropriate collection, transportation, or handling.

Logistic issues:

Issues such as a deficient number of laboratory, inadequate instrument, poor quality instrument and considerable cost of laboratory testing create the challenges in the performance of RT PCR. (3) If the availability of supplier of the single manufacturer will be many, these platforms will be costly, also a requirement of a high-level skilled technician and laboratory equipped with the necessary instrument. (2)These platforms with the above facilities will be only suitable for national or reference laboratories. Due to the monopoly of a single manufacturer, multiple numbers of tests cannot be performed at a district laboratory and clinic level. (2,3) Therefore, a clinic or laboratory capacity for the performance of tests must be increased and it **will be** possible without the monopoly of a single manufacturer. In remote and resource-deficient settings, easy procedure of testing and low-cost strategies is needed to support the full implementation of viral test like RT PCR. (2)

Implementation of rapid RT-PCR in the pandemic of COVID-19 has to be done at such places where scarcity of laboratory instruments is present. RT-qPCR requires expensive equipment such as RNA extraction instruments, rotor, and real-time PCR thermal cyclers, which are not available in many rural settings. Moreover, limitations are faced by even well-equipped labs due to minute difficulties from any unavoidable factor. (6)

The material used for RT PCR:

The market can face supply challenges and shortages of viral RNA extraction kits and sample preparation reagent in the age of the COVID-19 pandemic.

PCR Tubes: Polypropylene tubes free from enzymes like DNase, RNase, and pyrogen should be used by laboratories. The manufacturer of the thermocycler recommends the dimensions of PCR tubes or reaction plates. The selection of tubes should depend on the block and lid height. Thin-walled tubes are the best for heat transfer within the shortest amount of time which develops specificity and reproducibility. (6,7)

Gloves: Gloves should be disposable and easily accessed in each section of laboratories used during PCR analysis. Gloves can protect the technician from exposure to chemicals as well as Contamination of sample due to human DNases and RNase can be avoided by using gloves.(6)

Pipette tips: PCR analysis can be done with special tips preferably the barrier tips and aerosol-resistant tips. During pipetting, these tips reduce cross-contamination of samples. Purchase of pre-sterilized and pre-loaded tips in hinged racks provide tip protection and easy access. (6)

Transportation:

Transportation of the sample should be maintained with cold chain. Different kind of specimen during transportation will be stored in various ways. Molecular testing of samples is unsuitable and untrustworthy in a remote setting that is situated at a long distance from laboratories or clinics and does not have a daily transport network. (2) Transportation of particular samples remains one of the biggest challenges in a rural area. In transportation, time of collection, and time needed to reach the laboratory is the most important key factor for RT PCR samples. Storage mainly is another factor that affects the result of RT PCR. (2) The sample container should be filled with viral transport medium (VTM) in the proper amount during transportation. (2)

Outwear:

Clean laboratory cloths decrease the chances of contamination with a template or amplified nucleic acid. But in villages, the scarcity of clean and not portable water is found for all seasons. So it becomes impossible to proceed with routine laundering in the washing machine with detergent to clean the laboratory cloth. There is a need to take precautions while washing clothes. Laboratory cloths should be divided into two categories based on the risk intensity of the sample of disease. Cleaning of laboratory coats is mandatory for the reduction of the probability of contamination of the nominated workspace and the PCR reaction. (6-8)

There are specialized companies for the cleaning purpose of laboratory coats. But in villages, there is no such facility. The quantity of PCR work the performance of laboratories decides the cleaning density. With the ongoing pandemic of Covid-19, PCR laboratories are continuously busy with sample checking. Cleaning of Laboratory coats which are used for the preparation of positive controls should be cleaned more frequently and these coats should be used only for specific work of preparation of positive controls. Disposable laboratory coats also may be used for the elimination of the need for cleaning laboratory coats. (6-9)

The prevalence of enzymes on the skin is introduced with used laboratory clothes and gloves, which degrade the nucleic acid in the lab or contamination occurs and PCR gives false results. Importantly, No one can assure the sensitivity and specificity of the real-time RT-PCR test. To avoid inconsistency in results, different sample types can be used during different stages. (6)

Power Supplies:

The unreliable power supply is a big question mark for villages in India from past years. A specific quantity of voltage supply is decided for villages. In case of a break in the power supply, the necessity of power backup for laboratory setup. Laboratory setup for RT PCR

includes instruments like deep freezer (-20°C), PCR station, laminar airflow, centrifuge machine, etc. which requires high voltage. (2-4)

Voltage and electric current readings should be provided by power supplies. Electrodes should be checked before use to ensure that the fitting is preferably more permanent. Examination of electrical cables, electrode wires, and cable connections should be done for any kind of breakage, worn-out rubber, corrosion, or detachment. The work can get interrupted due to all these reasons and repairing can be done with the only high skilled engineer who is not available in a rural setting. (3-6)

Pipette:

Small volume transfers are involved by pipette in PCR methods. Calibration and handling of pipette are the most important factors for RT PCR success. Minor defects in pipette handling can create problems in the result of RT PCR. Therefore, handling of the pipette is possible with only a skilled laboratory technician. (8,9) If the persistence of problem with the pipette occurs in the rural area, the process to send the pipette for calibration or any repair will be lengthy and will take time to reach up to the manufacturer of the pipette. Sterilization of pipette can be done as per the manufacturer's recommendations regularly. The pipette is calibrated after sterilization. (4-6)

Disposal for reagents:

For RT PCR, there is a need to dispose of reagents very carefully. Some reagents can produce harmful effects on the ecosystem. Genetic changes occur due to entry into the ecosystem at any level. The PPE used in the laboratory must be bagged and prepared for disposal via the chemical hazardous waste stream. The pipette tips, sample tubes, PCR tubes, and gloves are the disposable materials utilized for the RT PCR which should be taken care of in the disposal process. (6,9)

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An alternative method to RT PCR for resource-limited setting:

Martzy et al suggested the loop-mediated isothermal amplification (LAMP) technique instead of RT PCR in remote areas. (9)

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Discussion:

Matthew Binnicker discussed the issues related with RT PCR during pandemic of Covid-19. Controversies or diagnostic questions arise during pandemic of Covid-19 was addressed by author. (1)

Teri Roberts et al believes that Investigation of such tests is required to work better in various type of setting and at a different level of the healthcare system.(1) Currently, tests conducted at national levels are very complex. Further simplification of these tests will be helpful for decentralization and easy to perform at the district level. Price reducing strategies are also required to carry full execution of RT PCR for viral load monitoring in a resource-limited setting. (2)

John Bartlett and John Shao have found that new diagnostic techniques can be evaluated and it will create a great opportunity for the development of laboratory infrastructure and training to modify the lab technician and other health staff. These benefits will refine service delivery for low and middle-income countries. (3)

Jane Greig et al have found that no one can assure the accuracy of results of viral load testing in a rural setting because of many reasons like complex equipment supplied by agencies, shortage of highly qualified scientific staff and service engineers, wastage of reagents in performing repeat test runs, etc. (4) A laboratory cannot provide accurate results because of support from the reputable international laboratory.

Arunkumar Arumugam et al have demonstrated that the use of unprocessed samples can be a feasible option to fasten the testing of RT PCR during a covid-19 pandemic. Time can be saved by using the unprocessed sample for RT PCR. Sample extraction time can be used to proceed with another important chore. Both material and human resources can be diverted with the approach of saving time, it might become a suitable pool for testing Covid-19 samples. To become convenient and approachable for the globe, this discovery can be helpful for rapid Covid-19 testing. (5)

Wei Feng et al have discussed the critical research needs raised during Covid-19 such as improvements in RT PCR and some issues related to RT PCR of Covid-19.(6)

Anil Giri et al described the problems faced by developing countries in the detection of covid-19. There was the unavailability of instruments, testing kits required for diagnosis, skilled human resources, Biosafety labelling, etc. (7)

Shuqi Wang et al have found that diagnosis-related with the point of care performs a key role for clinical investigation, treatment observation, prediction of prognosis, and disease surveillance on a large population level. For resource-limited setting, point of care testing assay becomes critical. Joint efforts from clinicians, biologists, and engineers will appear to enable required POC testing for RLS. (8)

Roland Martzy et al have demonstrated the use of other molecular techniques rather than RT PCR for the avoidance of complicated equipment. By combining LAMP and HDA like DNA amplification methods to an entire workflow, the analysis time is decreased from 6 hours to less than 2 hours. There is no necessity for a fully equipped molecular biological laboratory. The researcher has performed these processes by using only a pipette and a simple portable heating block instead of a laboratory (9). Few related studies on Covid-19 were reviewed (10-15).

The improvisation in outlook and reduction in faulty results can be achieved by the correct procedure for sample collection, standard practice for good laboratory, and use of superior isolation and real-time RT-PCR kit. The findings for RT PCR will help COVID-19 testing for more reliable and accessible results and can avoid the mistakes during the process all over the globe.

Conclusion:

RT PCR like diagnostic techniques and assays become decisive for clinical diagnosis and strategies planned to control the spread of Covid-19. SARS Cov-2 is a causative virus for the outbreak of Covid-19. Millions of people are infected by the Covid-19 virus around the globe

and cases of covid-19 have risen day after day. There are major opportunities for chemists to collaborate with doctors, researchers, health professionals and engineers and manufacturing of vaccine or any solution of accurate diagnosis of Covid-19. They can also improve molecular testing for SARS Cov-19.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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