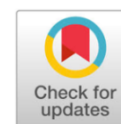


**Short Communication*****D18S51 A brief history of RT-PCR and our laboratory experience with SARS-CoV-2 analyses using RT-PCR*****Naci Omer Alayunt**

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Abstract: Polymerase Chain Reaction (PCR) is in vitro replication that allows accelerated amplification of certain sequences in small DNA fragments. A sensitive technique, only traces of DNA are needed for PCR to produce enough copies to be analyzed. In molecular diagnostic laboratories, rRT-PCR technique is applied to find target RNAs for the diagnosis of specific pathogens. Although the rRT-PCR method, which has high specificity and moderate sensitivity, is accepted by WHO as the gold standard test for the confirmation of COVID-19, there are many negative comments about this method that should be considered. While diagnosing SARS-CoV-2, it is possible to say that real-time PCR (RT-PCR) analysis is still valid but not sufficient to quickly distinguish similar infections. For this reason, there is a need for new analysis methods and new RT-PCR studies to be performed with newly developed unique rapid tests.

Keywords: Analytical error; COVID-19 virus; General protocol; Test kit; rRT-PCR.

INTRODUCTION

While studies on Deoxyribo Nucleic Acid (DNA) continue, PCR was first discovered by Kary Mullis in 1985. This technique, with its high sensitivity and specificity, has led to the evolution of diagnostic and research possibilities and has been awarded the Nobel Prize.¹ PCR is in vitro replication that allows accelerated amplification of specific sequences in small DNA fragments.² PCR; It has been applied in various fields such as biotechnology, cell biology, genetic engineering, forensic science, medical science, drug research. Methods for the efficient performance of PCR have been precisely optimized and have improved considerably over the past three decades.³ High sensitivity and specificity of PCR; It allows the detection of rare microorganisms in diagnostic clinical applications, especially in body fluid infections. It is also a method that detects organisms in a sample faster, cheaper and more accurately compared to culturing. In recent years, it has been observed that multiple (multiplex) PCR technique, which identifies and distinguishes more bacteria than traditional urine culture, and enables direct urine analysis, has been applied in patients with urinary tract infection symptoms.⁴ A sensitive technique, PCR only needs traces of DNA or RNA to produce enough copies to be analyzed. PCR can be performed after obtaining DNA from various tissues and organisms, including peripheral blood, skin, hair, saliva, and microbes.⁵

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PCR Steps

In PCR; template DNA, four deoxyribonucleotides (dNTPs: dATP, dTTP, dGTP and dCTP), two primers or oligonucleotides, DNA polymerase enzyme, buffer solution and magnesium (Mg^{+2}) incorporated into nucleotides to be recognized by the polymerase enzyme, responsible for making the new strand from the template DNA; The template is subjected to a series of thermal cycles to reproduce millions of copies of DNA.⁶ This cycle is basically the process that includes three steps: 1. Denaturation of the double-stranded DNA template, 2. Binding of target-specific primers, 3. Extension of bound primers by DNA polymerase.⁴ The method is performed at a temperature between 94°C-96°C for 1 minute to 10 minutes, depending on the template DNA and polymerase type. This is followed by the denaturation step, typically carried out at a temperature between 93°C-98°C. Hydrogen bonds in double-stranded DNA (dsDNA) are broken, resulting in two single-stranded DNA (ssDNA) molecules from each dsDNA (denaturation step). In the binding step, the temperature is then lowered to the primer-specific binding temperature in the range of 55°C to 65°C, so that the primers bind to complementary sequences of single-stranded DNA molecules. The PCR mix is then heated to a temperature between 72°C-80°C, depending on the polymerase used. During the elongation step, the incomplete DNA sequence is extended by polymerase in the presence of free dNTPs that synthesize new double-stranded DNA, which is a copy of the original DNA template.³

PCR Optimization

Problems such as the presence of inhibitory substances in the samples, the risk of environmental contamination, incorrect use of the amount of components used, and the inability to adjust the temperature parameters are always the problems that can be encountered during PCR. In addition, the design of oligonucleotide primers is only possible with known strains of microorganisms and known sequences of these strains. Another factor that can cause problems in the functions of PCR is unexpected mutations in microbial genomes. One of the most important problems that may be encountered in routine PCR applications in diagnostic laboratories is false positives due to contaminations. This problem shows that the laboratories where PCR will be performed must be strictly controlled. Apart from the problems that we may encounter during the routine control and evaluation of PCR, there are standards and rules that should be known for the optimization of the reagents and materials to be used. Briefly, the procedures to be followed are the standards and parameters used in optimizing the amount of reagents used and the quality of the materials; DNA Extraction, Mg^{+2} Concentration, primers, dNTPs, temperature parameters used in the PCR reaction, PCR machines and tubes in which the PCR reaction is carried out.

Real-Time PCR

Real-time PCR is a highly preferred method today.⁷ RT-PCR allows the target to be quantified relative to a calibrator and therefore the method is quantitative (qPCR).⁸ qPCR represents an enhanced version of standard PCR. With this technique, products are continuously monitored throughout their reaction cycle using fluorescent dyes. starting amount of DNA sequence; It can be generated by comparing the fluorescence output

curve of the qPCR with the standard curve produced with known different starting numbers of DNA copies. The threshold cycle (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold and be detected. Ct levels are inversely proportional to the amount of target nucleic acid in the sample.⁶ The sample output of real-time PCR is fast, and it is more sensitive and specific than conventional methods.⁷ qPCR is widely applied in clinical settings and remains the gold standard for nucleic acid measurement.⁸ Today, due to its high sensitivity level, qPCR technique is frequently used to detect malignant cells in different types of hematological malignancies.⁶

RT-PCR and SARS-CoV-2

PCR is accepted as a highly sensitive laboratory technique that can provide qualitative and quantitative results, and its reliability has been proven in the fields of medicine and biology (Figure 1). In molecular diagnostic laboratories, rRT-PCR technique is applied to find target RNAs in the diagnosis of specific pathogens.⁹ To diagnose SARS-CoV-2, although CT scan and other biochemical findings seem helpful in the diagnosis of COVID-19, as revealed in previous studies, they may have similarities with some infections with similar symptoms.^{10,11} The gold standard method that can distinguish SARS-CoV-2 from other beta-coronaviruses such as SARS and MERS for molecular diagnosis using specific primers and probes is the rRT-PCR method.¹²

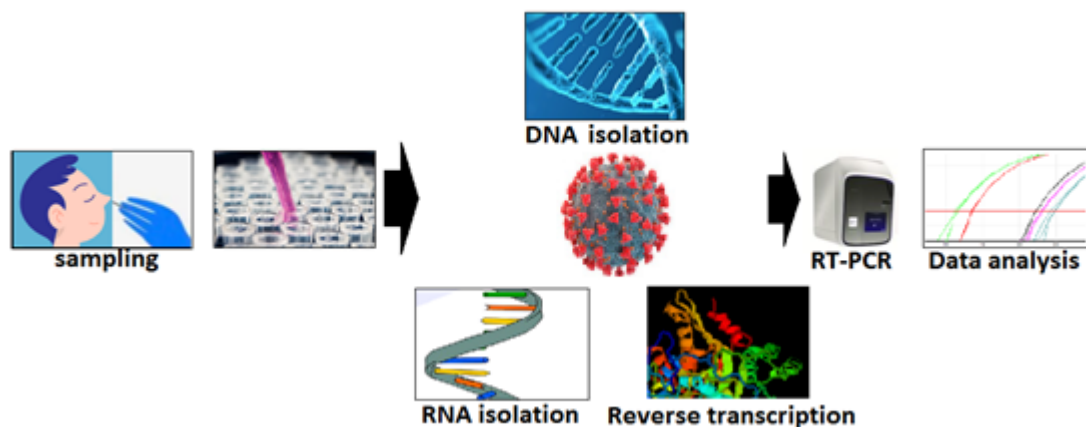


Figure 1. Working procedure of RT-PCR

Even though the rRT-PCR method, which has high specificity but moderate sensitivity, is accepted as the gold standard test for the confirmation of COVID-19 by WHO, there are many negative comments that should be considered on this method (Figure 1).¹³ Considering the pre-analytical errors; Test results may be affected in the steps until the samples are taken and finalized in the laboratory.^{14,15} Factors during analytical testing such as nucleic acid extraction, cDNA synthesis and PCR processing, and finally analytical errors such as interpretation and analysis of results and assay have also been reported.^{13,15,16} In general, rRT-PCR troubleshooting pre-analysis, pre-analysis, and post-analysis phases, and by following the guidelines, it is possible to effectively increase the accuracy and precision of the results obtained.

RESULTS AND DISCUSSION

Based on the detection of SARS-CoV-2 virus RNA isolated from upper respiratory secretions, RNA copies per throat swab sample, virus RNA concentrations were readily isolated from throat or lung-derived samples.¹⁷ Consistent diagnosis of COVID-19 is supported by viral tropism and high active replication rate in the pharyngeal region, but RNA isolation from blood, urine and stool samples is not preferred.¹⁷ Diagnosis of SARS-CoV-2 infection is currently based on real-time reverse transcriptase-polymerase chain reaction (RT-PCR) performed on nasopharyngeal swabs (NPS) or oropharyngeal swabs (OPS).¹⁸ Even though the diagnostic rates are not optimal with sample collection from the upper respiratory tract, it still represents the primary diagnostic method of COVID-19 patients with its NPS/OPS ratio.¹³ Some studies mention that RT-PCR results for COVID-19 infection are false-negative and will be a non-negligible error, especially for symptomatic individuals suspected of being infected with COVID-19.^{19,20} The use of CT to diagnose COVID-19 is known to be of great value in evaluating the course of the disease and treatment protocols. China uses CT instead of other research tools in the diagnosis of COVID-19, and the ability of CT to diagnose patients at an early stage may also be due to concerns about the specificity of other tests and the lack of virus test kits.²¹ However, due to the low specificity of CT in distinguishing COVID-19 from other similar diseases, the American College of Radiology (ACR) opposes the use of CT for the diagnosis of COVID-19 disease in the first place.²² In addition, the Royal College of Radiologists (RCR) state that CT has a very important role in the evaluation of patients with worsening clinical picture and severe respiratory distress, but that CT should not be used in the evaluation of coronavirus infection.²³ Ventilation, airflow and cleaning of scanner rooms, and other hygiene-related challenges in radiology areas are another reason not to view CT as the sole diagnostic tool for COVID-19 patients. Despite all this, it is noteworthy that RT-PCR has a low sensitivity (60-71%) compared to CT in the diagnosis of COVID-19 infection.^{20,24,25} Studies supporting the high sensitivity of CT images (98%) compared to RT-PCR tests (71%) are frequently encountered.²⁵

It has been reported that 3% of the patients from 167 people from whom nasopharyngeal and/or throat swabs were taken initially showed negative RT-PCR, but they were positively compatible with COVID-19 in simultaneous chest CT scans.²⁰ Multiple peripheral ground-glass opacities (GGO) can be observed in lingual segments known to be negative for RT-PCR laboratory test with a chest CT scan.¹⁹ Such false negative results can be explained by the low viral load and/or laboratory errors in the samples.^{20,25} The inadequacy of test kits may lead to the victimization of the patient and failure to detect similar errors again.²⁴ Therefore, more work falls on radiologists to diagnose COVID-19 (22). Wu et al. (2020) mentioned the role of chest CT scans in assessing the severity of COVID-19 infection, citing the fact that most patients had mild symptoms and a high fever, but the severity of lung findings on chest CT scans.²⁶

The studies may suggest that RT-PCR testing alone is not sufficient to prove the diagnosis of COVID-19. Therefore, early chest CT scans may still be necessary, along with other research tools such as RT-PCR

testing. Considering the psychological status of COVID-19 patients and healthcare workers during diagnosis and treatment, it is clear that large-scale new studies are needed on the reliability of RT-PCR results.²⁷

CONCLUSION

Although PCR-based methods, which are renewed with continuously developed tools, materials and ready-made kits, were initially developed for diagnostic purposes, they are currently used in many disciplines and fields. PCR-based methods, which require specialized molecular workers to be optimized, cause difficulties in laboratories until the optimization stages and can cause time and material loss. Even with repeated studies using information from optimized literature, the brand of materials and tools, their conditions of use, their misuse, and repeated reactions with inexperienced personnel may not yield the same results.

Even with the same instruments and brands, different results are obtained between different laboratories with experienced personnel. Even with all this in mind, the PCR technique continues to be an increasingly important and practical technique in diagnostic microbiology and other fields, despite its disadvantages and difficulties. This technique will continue to develop with increasing momentum in the coming years, with the PCR methods being renewed every day. In the current literature on COVID-19, although chest CT scans show high sensitivity in diagnosing COVID-19 compared to RT-PCR tests, chest CT scans alone are not sufficient to detect COVID-19.

The sensitivities of the RT-PCR tests in use are not sufficient to diagnose and guide the treatment of COVID-19. Judging from these research results, it is clear evidence that RT-PCR analysis is still not sufficient for the diagnosis of COVID-19 and that imaging methods and serum antibody tests should also be used. Considering the time, place and financial appropriations for the diagnosis of COVID-19; There is a need for RT-PCR studies to be conducted with newly developed unique tests.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The author report there are no competing interests to declare. The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors.

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