Original Article

Althea Medical Journal
ISSN Online: 2337-4330
https://doi.org/10.15850/amj.v11n4.3441

Concordance between Isothermal Amplification-Real Time Fluorescence Assay and Reverse Transcriptase Real Time Polymerase Chain Reaction for Detecting SARS-CoV-2

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Abstract

Background: There are various nucleic-acid-based diagnostic tests used to detect SARS-CoV-2 in COVID-19 patients such as reverse transcription polymerase chain reaction (RT-PCR) and isothermal amplification real time fluorescence assay (IARTFA), with the first-mentioned is considered as the gold standard. The IARTFA is an alternative method using cross priming amplification (CPA) technique under constant temperature, with a simpler procedure and faster result. This study aimed to determine the concordance between IARTFA and RT-PCR in detecting SARS-CoV-2.

Methods: This was an observational cross-sectional study with a random sampling method, conducted from August–October 2021 in Dr. Hasan Sadikin General Hospital, Bandung, Indonesia. Data on patients was obtained from medical records. Cohen's kappa test was used to determine concordance between both methods.

Results: A total of 30 subjects were collected, including subjects with positive result on RT-PCR and IARTFA (n=15), negative result on both methods (n=5), and with positive result on RT-PCR but negative on IARTFA (n=10). There were discrepancies between both methods in subjects with cycle threshold (CT) values ≥35. However, there was a significant concordance between both methods (Cohen's kappa coefficient of 1, p=0.006).

Conclusion: In addition to RT-PCR, IARTFA is an alternative method for detecting SARS-CoV-2 with advantages and excellent concordance. In communicable diseases such as COVID-19, along with healthy lifestyles such as isolation and wearing masks, early diagnosis is important to prevent and manage the spread of the disease.

Keywords: Cross priming amplification, isothermal amplification real time fluorescence assay, RT-PCR, SARS-CoV-2

Althea Medical Journal.
2024;11(4):235–240

Received: August 11, 2023
Accepted: February 16, 2024
Published: December 30, 2024

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Introduction

At the end of 2019, an outbreak of a coronavirus disease later known as Coronavirus disease 2019 (COVID-19) emerged in Wuhan, China. This outbreak was caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and turned into a pandemic that created problems for the world's health system. Therefore, early diagnosis, supportive care, and isolation of infected individuals and their contacts are crucial to control the current pandemic.[1]

The genome of the virus responsible for COVID-19 is composed of a single-stranded RNA molecule containing the genetic information necessary for replication and infection of host cells. The genome of SARS-CoV-2 is approximately 30,000 nucleotides long and encodes various proteins, including the Spike (S) protein which is located on the surface of the virus and is responsible for binding to the angiotensin-converting enzyme (ACE2) receptor. This binding facilitates entry of the virus into host cells. Other proteins include the envelope (E) protein, the

membrane (M) protein, the nucleocapsid (N) protein, RNA polymerase, and other proteins.[2]

Currently, reverse transcription polymerase chain reaction (RT-PCR) is the gold standard for COVID-19 detection, which is based on nucleic acid amplification.[3,4] This RT-PCR method has a diagnostic specificity of almost 100%.[5] The RT-PCR test is highly specific, but its sensitivity depends on the amount of virus, viral replication, as well as the source and time of sampling related to the disease phase.[6] The RT-PCR test transcribes and amplifies the specific genome sequence of SARS-CoV-2.[4] Another diagnostic method based on amplification for detecting COVID-19 is the isothermal amplification real-time fluorescence assay (IARTFA), an advancement in molecular technology.[7,8]

Cross priming amplification (CPA) technology is used in the isothermal amplification tests, which do not require a thermal cycling program.[9,10] While retaining the same level of sensitivity, the isothermal detection methods provide considerable advantages over RT-PCR systems in terms of cost, speed, and labor savings. Only a few studies support this test as an alternative for detecting COVID-19. This study aimed to determine the differences between IARTFA and RT-PCR as the gold standard in detecting SARS-CoV-2 at Dr. Hasan Sadikin General Hospital, Bandung, Indonesia.

Methods

This study was carried out at the Laboratory of Molecular Biology, Dr. Hasan Sadikin General Hospital, Bandung, Indonesia, from August to October 2021. This study was a cross-sectional observational study and used the Cohen's kappa consistency test. In total, 30 data on sample patients (nasopharyngeal or oropharyngeal samples) were obtained randomly from medical records. The inclusion criteria were samples with negative and positive results with a CT value ≤38 of RT-PCR (Biosewoom). The exclusion criteria were incomplete patient data. This study protocol was approved by The Research Ethics Committee of Dr. Hasan Sadikin General Hospital Bandung (No: LB.02.01/X.6.5/299/2022).

The research procedure began with the RT-PCR test, followed by the IARTFA test (UStar EasyNAT®) consecutively on the same day. The results obtained were cycle threshold (CT) in RT-PCR, and time threshold (TT) in IARTFA. Both methods were interpreted with positive and negative result.

Table 1 Subject Characteristics (n=30)

Variable	n
Gender	
Male	18
Female	12
Age (year)	
<18	3
18–25	5
26–35	8
36–45	4
46–55	7
56–65	2
>65	1
Patient status	
Inpatient	11
Outpatient	19

The data obtained was calculated for suitability using the Cohen's kappa (K) consistency test with the following interpretation criteria: poor (K<0.2); fair (K 0.21–0.40); moderate (K 0.41–0.60); good (K 0.61–0.80), and very good (K 0.81–1.00). Analysis was processed using the SPSS Version 25.0.

Results

Of the 30 data collected, males were more prevalent (18 of 30), and most in the age group of 26–35 years (8 of 30) old. The majority were outpatients (19 of 30) (Table 1).

There were 15 subjects positive for RT-PCR and IARTFA, 5 subjects negative for RT-PCR and IARTFA; and 10 subjects positive for RT-PCR but negative for IARTFA. Cohen's kappa coefficient was 1, indicating that there was a significant similarity with very good criteria between IARTFA and RT-PCR with a p-value of 0.006 (Table 2).

Discussion

The RT-PCR method is widely used as the gold standard for diagnosing COVID-19 and requires the extraction of RNA. This method is relatively time-consuming, which is a significant limitation in situations where rapid decision-making is crucial. Moreover, it requires specialized equipment and trained personnel, limiting its availability in resource-limited settings. The cost of RT-PCR tests can be high, especially when considering the need for specialized equipment, reagents used, and trained personnel. This can limit its accessibility,

Table 2 Concordance of Isothermal Amplification Real-Time Fluorescence Assay Results compared to RT-PCR

Procedure	Total	RT-PCR		Kappa	p value
		Positive	Negative		
Isothermal amplification real-time fluorescence assay				1.00	0.006
Positive	15	15	0		
Negative	15	10	5		
Total	30	25	5		

Note: RT-PCR= reverse transcription polymerase chain reaction

particularly in low-income countries or regions with limited healthcare infrastructure.[11] The RT-PCR method remains the most reliable and accurate method for diagnosing COVID-19, particularly for confirming cases and guiding clinical decisions; however, these limitations highlight the need for complementary testing strategies, especially in certain contexts like mass screenings or in areas with limited laboratory capacity.[12]

On the other hand, diagnostic tests utilized for surveillance aims are specifically developed to evaluate the occurrence of SARS-CoV-2. These tests should possess cost-effectiveness, swift execution, and simplicity in administration, facilitating repeated testing. Recent advancements in diagnostic testing have led to the development of innovative virological and serological assays specifically designed for rapid detection of SARS-CoV-2, the causative agent of COVID-19, at point-of-care (POC) locations. These novel diagnostic methods are pivotal in streamlining the detection process, particularly in limited time conditions. The important point in virological diagnosis involves the direct detection of viral nucleic acids, which is achieved through the application of isothermal nucleic acid amplification tests (iNAATs).[13] Furthermore, the subsequent development of CPA method has added another dimension to the specifications of diagnostic tools. The CPA method operates on the principle of strand displacement activity facilitated by DNA polymerase.[9]

Cross-priming amplification (CPA) represents a novel technique for DNA amplification, functioning optimally at a steady temperature of 63°C. This method distinguishes itself from other amplification techniques by eliminating the necessity for the traditional cycling of temperatures. The CPA incorporates several interlinked primers, typically in the range of six to eight, which negates the requirement for a preliminary denaturation phase or the use of a nicking enzyme.[9]

During the CPA process, the maintenance of a constant temperature induces the spontaneous creation of denaturation bubbles within the DNA's double strands. This phenomenon increases the availability of single-stranded DNA, thereby enhancing the primer's ability to attach to the template at these transient denaturation points. The process of strand displacement is initiated by cross primers, characterized by its 5' end being non-complementary to the template strand, along with the incorporation of a displacement primer situated upstream of the cross primer. One of the defining features of CPA, setting it apart from other iNAATs, is the use of one or more cross primers. These primers are uniquely designed with a 3' end complementary to the target sequence and a non-matching 5' end, which includes an additional sequence. In this phase, the DNA polymerase extends the primer-template complex, facilitating a rapid multiplication of the target DNA. The CPA methodology is particularly notable for producing up to eight different amplicons, which are characterized by their high specificity and sensitivity.[14]

This study has shown that male, those aged between 26 and 35 years, and outpatient received more RT-PCR tests. This is consistent with data on the official website of the Indonesian government managed by the Task Force for the Acceleration of Handling COVID-19 which states that of the 8,211 cases of patients diagnosed with COVID-19, men in their prime working years make up the most of the COVID-19 epidemic. Men are more mobile than women, which contributes to the high incidence of COVID-19 cases in men, and men were also naturally more vulnerable to the virus.[15] Men are more genetically to exhibit ACE-2 receptors, which is more important in COVID-19 infection.[16,17]

The results showed that 10 subjects were positive with the RT-PCR test but negative with

the IARTFA test. This was found in all subjects examined with RT-PCR with a CT value ≥35. The results of the study indicate that the CPA assay demonstrates considerable efficacy in detecting cases of individuals who have either a high or moderate viral load of SARS-CoV-2. However, its performance appears to be less effective in low viral load cases, such as during the initial stages of infection or in the late stages of the illness.[18] This reduced sensitivity in detecting SARS-CoV-2 infection in low viral load scenarios, particularly in instances where the CT value was above 30, could be linked to the difference in the LoD between the CPA method and RT-PCR.[19]

A critical observation in this context is that the CPA assay exhibits a higher LOD, quantified at 103 copies of the virus, compared to the RT-PCR method, which has a LOD of only 10 copies.[20] This variance in LOD is a key factor contributing to the differing sensitivities of these two methods, especially in detecting lower concentrations of the SARS-CoV-2 virus. Consequently, while CPA is reliable for detecting substantial to moderate viral loads, its lower sensitivity in low viral load situations is a notable limitation.[21] The Ustar EasyNAT has a detection limit of 200 copies/mL.[22] In addition, discrepancies may occur due to differences in methods and principles of test between RT-PCR and IARTFA.

The RT-PCR involves multiple stages, starting with RNA extraction and precise thermal cycling. This cycle repeats three times until reaching a predetermined cycle number. The Biosewoom kit comprises essential reagents such as PCR reaction mixture, RT-PCR enzyme, primers, probes, positive and negative controls, and sterile water. Specific oligonucleotide primers from the RdRP and E genes target SARS-CoV2, with a human RNase P (HRP) control. Different fluorescence labels (FAM for RdRP, HEX for E gene, and Cy5 for HRP) distinguish the amplified genes. Results are usually obtained within 2–3 hours, interpreted qualitatively as positive or negative, with a positive result requiring a CT value ≤38, a threshold limit of 300 RFU, and a detection limit of 6.25 copies/mL.

In contrast, the IARTFA method uses cross-priming amplification (CPA) with constant temperature conditions. It operates within a closed cartridge, divided into three chambers for lysis, washing, and amplification. The process starts with adding COVID-19 RNA extraction solution and the sample into the cartridge, enabling automatic RNA purification. Hydrophobic separator layers in the cartridge isolate components of the reaction. Extraction occurs at 95°C, followed by washing at 75°C, and the nucleic acid moves to the amplification chamber. There, specific primers, fluorescent probes, and DNA polymerase amplify the target at a constant 58°C. The instrument detects fluorescence signals, generating a curve. An internal control (IC) monitors the entire process effectiveness. The IARTFA examination takes a total of 55 minutes, including 25 minutes for sample processing and 30 minutes for amplification. Results are interpreted qualitatively as positive or negative, with a positive cutoff at TT ≤30 and a detection limit of 200 copies/mL.

The RT-PCR uses extraction of RNA and thermal cycling, while IARTFA uses CPA with several (six to eight) primers and a template multiplication under constant temperature to created exponential amplification.[23,24]

The IARTFA test offered the advantage of a quicker inspection time, resulting in a shorter turnaround time (TAT). Fifty five minutes are needed in total, including 25 minutes for processing the sample and 30 minutes for amplification.[22] With this intimate system, nucleic acid analysis can also decrease the possibility of contamination and simplify work processes which does not require a biosafety level-2 (BSL-2) facility.[25,26] Although, saliva samples can be evaluated, but a nasopharyngeal swab sample was the most strongly advised by the WHO.[27,28] The previous study showed that the CPA has good validity to detect SARS-CoV-2 infection with a sensitivity of 77%, specificity of 94%, and area under the ROC curve of 84%, suggesting that CPA could give 84% true diagnosis for detecting SARS-CoV-2 infections. The minimal cut-off AUC for the diagnostics test was 50%. Therefore, CPA could be used as rapid molecular testing for detecting SARS-CoV-2 viral RNA from pharyngeal swab specimens.[24]

A limitation of the study is the usage of a closed system, which prevents each stage from being evaluated and may have an impact on the test's outcomes of the isothermal method. It is recommended that each procedure from swabbing to inserting the kits, is followed to ensure the validity of the test.

In conclusion, this study shows that the IARTFA approach is an alternative method for detecting SARS-CoV-2 because it has advantages and very good agreement compared to RT-PCR. The isothermal method can be applied in public facilities, such as airports or hospitals to quickly identify symptomatic and asymptomatic individuals, thereby help to

reduce the spread of COVID-19. More studies are needed to examine the potential value of isothermal method in detecting various diseases.

In communicable diseases such as COVID-19, along with a healthy lifestyle such as isolation and wearing a mask, early diagnosis is important to prevent and manage the spread of the disease. The ease of access in IARTF assay may assist in establishing diagnose, facilitating early isolation for infected individuals.

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