Artikel Penelitian

Use of The Heat Treatment Method as An Alternative to Commercial Kit-Based Nucleic Acid Extraction Method for Detecting SARS-CoV-2 Using RT-qPCR Technique under Reagent Limited Conditions

Alhawaris[1], Yadi[1], Nataniel Tandirogang[1]

Abstrak

Peningkatan jumlah kasus COVID-19 dapat menyebabkan gangguan pada rantai pasokan global kit komersil untuk ekstraksi asam nukleat SARS-CoV-2 sehingga proses pemeriksaan COVID-19 berpotensi menjadi terhambat. Metode alternatif lainnya yang dapat digunakan adalah metode heat-treatment (HT). Penelitian ini bertujuan untuk menilai kualitas hasil pemeriksaan RT-qPCR untuk deteksi SARS-CoV-2 menggunakan metode ekstraksi asam nukleat HT. Sampel berupa hasil swab nasofaring dan orofaring kemudian melalui proses ekstraksi asam nukleat menggunakan metode ekstraksi magnetic beads (MBE) dan metode HT pada hari yang sama (kurang dari 6 jam). Hasil ekstraksi asam nukelat kedua metode kemudian diamplifikasi menggunakan teknik RT-qPCR, lalu dibandingkan dan dianalisis hasilnya. Hasil penelitian menunjukkan bahwa secara kualitiatif terdapat perbedaan yang sangat signifikan pada interpretasi hasil di antara kedua metode ekstraksi asam nukleat (uji McNemar, $p = 0$; $p < 0.01$). Analisis komparatif secara kualitatif juga menunjukkan bahwa terdapat perbedaan nilai ct yang sangat signifikan pada gen-gen SARS-CoV-2 yang dideteksi di antara kedua metode ekstraksi asam nukelat (uji McNemar, $p = 0$; $p < 0.01$). Sedangkan nilai ct pada gen manusia di antara kedua metode esktraksi asam nukelat secara kualititaif tidak terdapat perbedaan yang signifikan (uji McNemar, $p = 1$; $p > 0.05$). Penggunaan metode heat-treatment untuk ekstraksi akan lebih efektif pada sel-sel atau microorganism yang memiliki material genetik berupa DNA dibandingkan RNA. Metode HT tidak dirokemendasikan untuk digunakan dalam diagnosis COVID-19. Penggunaan metode ini sebagai screening pada populasi bergejala sedang hingga berat dalam kondisi keterbatasan reagen ekstraksi dapat dipertimbangkan.

Kata kunci: SARS-CoV-2, COVID-19, Metode Heat-Treatment, Ekstraksi Asam Nukelat, RT-qPCR

Abstract

The increase in the number of COVID-19 cases could cause disruptions to the global supply chain of commercial kits for the extraction of SARS-CoV-2 nucleic acid so that the COVID-19 screening process has the potential to be hampered. Another alternative method that can be used is the heat-treatment (HT) method. This study aims to assess the quality of the results of the RT-qPCR examination for the detection of SARS-CoV-2 using the HT nucleic acid extraction method. The samples were nasopharyngeal and oropharyngeal swabs and then underwent nucleic acid extraction using the magnetic beads extraction (MBE) / commercial kit and the HT method on the same day (less than 6 hours). The nucleic acid extraction results from both methods were then amplified using the RT-qPCR technique, then compared and analyzed the results. The results showed that qualitatively there was a very significant difference in the interpretation of the results between the two nucleic acid extraction methods (McNemar test, $p = 0$; $p < 0.01$). Qualitative comparative analysis also showed that there were very significant differences in ct (cycle threshold) values in the SARS-CoV-2 genes detected between the two nucleic acid extraction methods (McNemar test, $p = 0$; $p < 0.01$). While the value of ct in human gene between the two methods of nucleic acid extraction qualitatively there was no significant difference (McNemar test, $p = 1$; $p > 0.05$). The use of HT method for extraction will be more effective on cells or microorganisms that have genetic material in the form of DNA than RNA. The HT method is not recommended for use in the diagnosis of COVID-19. The use of this method as a screening in moderate to severe symptomatic populations under conditions of limited extraction reagents may be considered.

Keywords: *SARS-CoV-2, COVID-19, Heat-Treatment Method, Nucleic Acid Extraction, RT-qPCR*

Submitted : 10 June 2024 *Revised : 28 October 2024* *Accepted : 17 December 2024*

Affiliasi penulis : 1 Medical Faculty of Mulawarman University, Samarinda, East Borneo, Indonesia
Korespondensi : Alhawaris alhawaris@fk.unmul.ac.id
Telp: 085151440123

INTRODUCTION

COVID-19 (Coronavirus Disease 2019) is an often fatal acute respiratory syndrome caused by infection of the pathogen virus, SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2). SARS-CoV-2 is an RNA virus belonging to the beta coronavirus genus (1). Transmission of SARS-CoV-2 can occur between humans through droplets or

aerosols as well as through the fecal-oral route in susceptible populations (2).

As of June 19, 2022, more than 536 million confirmed cases of COVID-19 have been reported and more than 6.3 million of them have died globally (3). Since it was declared a global pandemic on March 11, 2020 (4) until mid-2022, the trend in the number of COVID-19 cases every year has always fluctuated. Within a year, the number of COVID-19 cases can increase several times in certain months. When COVID-19 cases increase, efficient and effective examinations are needed so that the spread of COVID-19 can be minimized and the epidemic condition can be controlled.

One of the recommended examination techniques for the diagnosis enforcement of COVID-19 is the nucleic acid amplification test (NAAT) by RT-PCR (Reverse-Transcription Polymerase Chain Reaction). Detection of SARS-CoV-2 nucleic acid by PCR technique is the gold standard for examination of COVID-19. It was first published and recommended by WHO on January 13, 2020 (4,5,6). RT-PCR technique is used to amplify genetic material in the form of RNA using reverse transcriptase to be converted into complementary DNA (cDNA). This cDNA was then amplified using PCR (7). The RT-PCR technique is then combined with the Real-Time PCR technique quantitatively or semi-quantitatively (RT-qPCR = Reverse Transcriptase-quantitative PCR) so that the detection of SARS-CoV-2 can run more efficiently, quickly, and accurately (8,9).

One of the important pre-analytical stages in the RT-qPCR technique for COVID-19 is the extraction of SARS-CoV-2 RNA. This activity aims to lyse cells, isolate, and purify SARS-CoV-2 genetic material before detection by separating unstable viral RNA from cell components or other contaminants (10,11).

Currently, there are many commercial kits available for the extraction of SARS-CoV-2 RNA, both for manual and automatic extraction of viral RNA. The magnetic beads extraction method (MBE) (12) or spin column (13) used in these commercial kits is relatively easier and faster to apply for the examination of samples on a large scale compared to the conventional method. However, an increase in the number of COVID-19 cases could cause disruptions to the global supply chain of these commercial kits, so the COVID-19 examination process carried out by many diagnostic laboratories has the potential to be hampered. Therefore, other alternatives are needed to anticipate this, one of which is by using the heat-treatment method (HT). This method is suggested by the Centers for Disease Control and Prevention (CDC) in the case of shortage of nucleic acid extraction reagents and urgent demand for SARS-CoV-2 detection tests (14). This method is simpler, faster, and relatively easier than using commercial extraction kits or other extraction methods. This study was conducted to assess the quality of the results of the RT-qPCR examination for the detection of SARS-CoV-2 using HT and to compare it with the extraction method using a commercial kit.

METHOD

Location and Sample

This sampling and research were carried out at the Microbiology Laboratory, Faculty of Medicine, Mulawarman University, Samarinda City, East Kalimantan, Indonesia which has been designated as a COVID-19 Examination Laboratory by the Ministry of Health of the Republic of Indonesia (decree number: HK.02.03/I/11103/2020, laboratory code: C.184). The research design is true experimental research.

The research sample used was nasopharyngeal and oropharyngeal swabs obtained from the population who visited the Laboratory to conduct an examination of COVID-19 using the RT-qPCR technique.

Method and Data Analysis

Nasopharyngeal and oropharyngeal swab samples obtained using flocked swabs were then placed in a VTM (Viral Transport Medium) tube. Samples were immediately examined on the same day (less than 6

hours). The entire process of sample management and nucleic acid extraction was recommended to be carried out at least in a Class IIA Biosafety Cabinet (BSC-IIA) in a Biosafety Level 2 (BSL-2) room. The extraction method used for the diagnosis of COVID-19 was MBE using the commercial kit *ZiXpress® 32 Viral DNA/RNA Extraction Kit*. The extraction process was carried out automatically using the *ZiXpress 32® Automated Nucleic Acid Purification Instrument* (once running maximum for 32 samples). Extraction using HT method was carried out using a thermal cycler machine as shown below (the scheme is adjusted to the availability of tools and materials at the research site) (14).

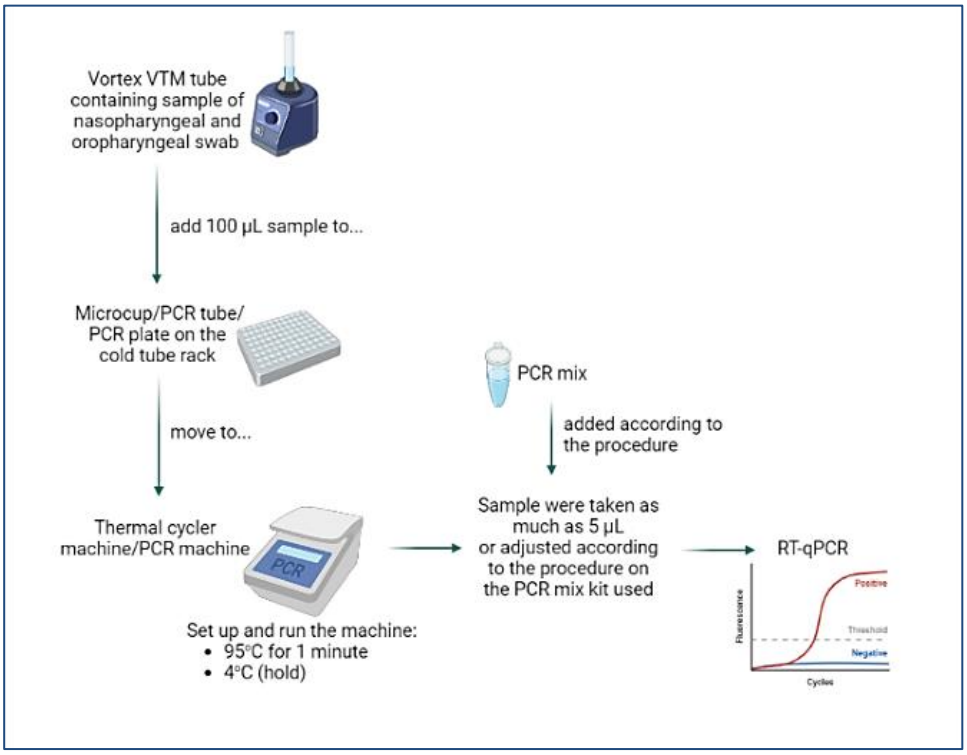

Figure 1. Workflow of Heat-Treatment Extraction Method (Created in BioRender.com)

The nucleic acid extraction products from both methods were then added with the same PCR Mix reagent (commercial kit; mBioCoV-19) to detect the presence of SARS-CoV-2 exposure in these samples. The viral genes detected using the PCR Mix reagent were the ORF1b (Open Reading Frame 1b)/helicase gene and the RdRP (RNA-dependent RNA Polymerase) gene, while the human gene (control) detected was RPP30 (Ribonuclease P Protein Subunit P30). The interpretation of the examination results is as shown below.

Table 1. Result Interpretation

ORF1b	RdRP	RPP30	Interpretation	Report	
+	+	+/-	SARS-CoV-2 detected	COVID-19 Positive	Amplification result is deemed positive if the ct value is less than 40 (< 40)
If only one is positive		+/-	SARS-CoV-2 detected	Inconclusive	
-	-	+	SARS-CoV-2 undetected	COVID-19 Negative	
-	-	-	Invalid	Invalid	

The RT-qPCR process was carried out using a *Rotor-Gene-Q* thermal cycler machine connected to a computer. The result analysis of the PCR process and the determination of ct values were carried out computerized using the *Q-Rex* program

which was connected to the thermal cycler machine. The data obtained were then analyzed using *SPSS Statistic 22*. The samples analyzed were all samples detected by both virus genes or one of them (positive or inconclusive) using MBE (commercial kit). The data from the analysis of these samples in the form of ct values using the commercial kit were then compared with the data from the analysis of ct values obtained through the HT method.

RESULT

The results of the RT-qPCR examination for some samples using the MBE method automatically obtained the number of positive samples (both viral genes were detected) and inconclusive (one of the viral genes was detected) as many as 50 samples. These data were then compared with the results of the RT-qPCR examination using the HT method. While the sample data with negative and invalid RT-qPCR results using the MBE method were not used.

Based on the data, the samples in this study consisted of 25 symptomatic patients and 25 asymptomatic patients. Samples obtained from asymptomatic patients were divided into 2: (1) Patients who had never done an RT-qPCR examination before (new patients; first examination) as many as 2 people, and (2) Patients who had RT-qPCR examination 2 weeks before (patients after self-isolation for 14 days; second examination) as many as 21 people.

The raw data obtained were grouped based on the results interpretation of the RT-qPCR examination and the ct value of the RT-qPCR examination results in the two extraction methods used (Table 2 and Table 3). Table 2 shows that 46 positive samples and 2 inconclusive samples were examined by RT-qPCR using the MBE method. The positive samples were compared with the RT-qPCR examination using the HT method and the results obtained were as follows: (1) Positive as many as 10 samples, (2) negative as many as 32 samples, and (3) inconclusive as many as 1 sample. All samples with inconclusive results using the MBE method obtained negative results when examined using the HT method. There were no samples with invalid results in both extraction methods. Qualitative comparative analysis using the McNemar test for both nucleic acid extraction methods obtained a *p*-value = 0 ($p < 0.01$) which means there is a very significant difference in the results of the RT-qPCR examination between the two methods.

Table 2. The Data Distribution of Interpretation of RT-qPCR Examination Result

Symptom	Examination	RT-qPCR Results								*p*
		MBE Method				HT Method				
		POS	NEG	INC	INV	POS	NEG	INC	INV	
		n				n				
Symptomatic	First Examination	23	0	2	0	9	10	6	0	0.000
Asymptomatic	First Examination	2	0	0	0	1	0	1	0	
	Second Examination	21	0	2	0	0	22	1	0	
Total		46	0	4	0	10	32	8	0	

POS = Positive; NEG = Negative; INC = Inconclusive; INV = Invalid
McNemar Test, p < 0.01

Table 3 shows the comparison of ct values between the two nucleic acid extraction methods. The ct value interval in table 4 is obtained from the data in the yellow column (Table 1) where the data compared is the samples detected using RT-qPCR in both extract methods for each of the examined

genes (viral genes: ORF1b and RdRP, human gene: RPP30).

Table 3. The Data Distribution of CT Value from RT-qPCR Examination Results of the Two Nucleic Acid Extraction Methods on Each Detected Gene

Gen	CT Value	Extraction Method			CT Value Interval Mean ± SD (Min – Max)	*p*
		MBE	HT			
		Detected (n)	Detected (n)	Undetected (n)		
ORF1b	< 21	5	5	None	7.1923 ± 2.18392 (2.80 – 9.97)	0.000
	21 – 30	27	8	19		
	31 – 40	17	None	17		
RdRP	< 21	5	5	None	5.4485 ± 3.13568 (0.14 – 9. 54)	0.000
	21 – 30	14	7	7		
	31 – 40	28	3	25		
RPP30	< 21	2	2	None	2.2178 ± 0.96045 (0.36 – 4.58)	1.000
	21 – 30	48	48	None		
	31 – 40	None	None	None		

McNemar Test, p ORF1b < 0.01; p RdRP < 0.01; p RPP30 > 0.05

Qualitative comparative analysis using the McNemar test on each detected genes obtained the results: (1) *p*-value of ORF1b and RdRP = 0.000 ($p < 0.01$); it means that there is a very significant difference in the ct value of the ORF1b and RdRP genes between the two nucleic acid extraction methods, and (2) the *p*-value of RPP30 = 1,000 ($p > 0.05$); it means that there is no significant difference in the ct value of the RPP30 gene between the two nucleic acid extraction methods.

DISCUSSION

The HT method is widely used as an alternative method for the extraction of nucleic acids (DNA and RNA) under limited reagent conditions using commercial kits (generally based on chemical or enzymatic principles). This method can be done by heating through boiling which is able to lyse cells through the destruction of cell membranes and protein denaturation. This method is efficient, simple, inexpensive, fast, and compatible with PCR (15,16,17).

The stability of the virus to heat varies widely. Viral surface proteins can be denatured within minutes at 55°C to 60°C. This can cause the virion to become non-infectious because it is no longer able to attach normally to host cell receptors (18). Enveloped viruses such as SARS-CoV-2 are more heat-labile than non-enveloped viruses and can be inactivated at 56oC for less than 30 minutes, 65oC for about 15 minutes, and at 95oC or above 75oC for about 3 minutes (18). ,19,20).

SARS-CoV-2 is a novel β-coronavirus composed of four structural proteins (Spike, Envelope, Membrane, and Nucleocapsid) and non-structural proteins (nsp1 – 16). Its genome is single-stranded positive-sense RNA (+ssRNA) (21,22). The emergence of genes encoding the formation of SARS-CoV-2 proteins through RT-qPCR examination is a marker of exposure to the virus in the body where the sample was obtained, such as the nasopharynx and oropharynx area.

Most of the samples diagnosed with Positive COVID-19 using the MBE method showed negative and inconclusive results when examined using the HT method. This shows that the SARS-CoV-2 genes in the form of RNA chains examined were not detected or only one of them was detected using RT-qPCR. Meanwhile, the human gene (RPP30) which is a DNA chain in both extraction methods can be detected well where the ct values between the two nucleic acid extraction methods are not significantly different. There are 3 factors that make this possible: (1) Stability of nucleic acids (DNA and RNA), (2) Presence of RNase, and (3) Inhibitors.

RNA contains ribose which is characterized by the presence of a 2′-hydroxyl group on the pentose ring. The hydroxyl group is the main reason that RNA is less stable than DNA because it is more susceptible to hydrolysis. Phosphodiester bonds in RNA chains are also much more labile than in DNA chains (23). Viruses that have genetic material ssRNA (single-stranded RNA) such as SARS-CoV-2 are more susceptible to inactivation than dsRNA (double-stranded RNA) viruses because ssRNA is relatively easier to hydrolyze than dsRNA (24). Another environmental factor that can affect the stability of RNA is the presence of the enzyme RNase (ribonuclease). These three classes of enzymes (endonuclease, 5'exonuclease, and 3'exonuclease) have activity in cleaving and degrading RNA structures. RNase II is a specific ribonuclease acting on ssRNA (25,26,27).

Nucleic acid extraction using commercial kits generally uses solid phase extraction methods, such as the spin column method (using silica particles) and MBE. This extraction method provides a nucleic acid purification process that is more effective than HT method and more efficient than conventional methods. This method can produce high purity genetic material by removing various other inhibitors through cell lysis, DNA or RNA precipitation using ethanol or isopropanol, binding, washing, and elution (28,29,30,31,32). Inhibitors such as cellular debris, microparticles, or other macroparticles other than DNA or RNA can interfere with the PCR amplification process (32). This may have implications for the interpretation of the results of the RT-qPCR examination for COVID-19.

The HT method is more effective in condition where there is a large amount of SARS-CoV-2 exposure in the sample shown through RT-qPCR examination using the MBE method with the ct value of < 20 in each viral genes where the patient shows symptoms of COVID-19. Meanwhile, samples with the ct value > 20 for each viral gene examined using the MBE method showed varied results (detected with a higher ct value or not detected) when examined using the HT method. This shows that the results of the RT-qPCR examination with the HT method can cause false negative values. In addition, the ct value is not absolutely correlated with the onset of symptoms in patients (33,34,35) so patients who appear to have clinical symptoms of COVID-19 do not necessarily show positive results with low ct until they are examined by RT-qPCR using the MBE method, especially if using the HT method. Therefore, this method cannot be used for the diagnosis of COVID-19.

CONCLUSION

The use of the HT method for nucleic acid extraction will be more effective on cells or microorganisms that have genetic material in the form of DNA than RNA. This method can cause bias or false negative results in the examination of SARS-CoV-2 exposure using RT-qPCR so it is not recommended for use in the diagnosis of COVID-19. The use of this method as a screening in moderate to severe symptomatic populations under conditions of limited extraction reagents may be considered. Further research is needed to find a nucleic acid extraction method that is simple, fast, easy, and compatible with PCR, for example by modifying or adding to the HT method.

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