Advances in Biological Sciences Research, volume 22
7th International Conference on Biological Science (ICBS 2021)

Comparison of Cycle Threshold Value of The Orf 1 ab SARS-CoV-2 Gene from Three Different PCR Reagents

Anita L. Susanti[1,*] Fusvita Merdekawati[2] Rohayati Rohayati[2]

[1]*Fakultas Kedokteran Universitas Jenderal Achmad Yani (UNJANI)*
[2]*Technical Laboratory Medicine Poltekes Kemenkes RI Bandung*
[*]*Corresponding author. Email: anitalee.dr@gmail.com*

ABSTRACT
The availability of PCR reagents is an obstacle that still needs to be resolved in the early days of the SARS-CoV-2 pandemic. During the early days of the pandemics, identical PCR reagents supply in the laboratory could not be guaranteed. The laboratory needs to investigate the reliability of the different reagents used for examination, to make sure that the results of the examination are valuable to patient management. This study aims to compare the Cycle Threshold (CT) value of the Orf 1 ab gene from three SARS-CoV-2 PCR reagents used for diagnosis, monitoring of the patient condition, and progress of therapy. The research sample was taken from nasopharyngeal and oropharyngeal swabs of individuals who were in close contact with confirmed SARS-CoV-2 patients. The reagents compared was originated from manufacturers in China and from South Korea. All of the examinations were performed with reverse transcriptase real-time PCR. The study was designed cross-sectionally. Comparative analysis for paired data was performed using Friedman's test and Wilcoxon's Post Hoc analysis. The results showed that the median CT value of the Orf 1 ab gene examined with reagents 1, 2, and 3 was 25,96; 24,87, and 28,39. The results of the analysis showed that there was a difference in the CT value of the Orf1 ab gene between the three PCR reagents ($p < 0.05$). The results showed that if CT value is to be used for diagnosis and monitoring of each patient's condition then the reagents used in the PCR examination cannot be replaced interchangeably.

***Keywords**: CT value, Orf 1 ab. Reagents, RT- PCR, SARS-CoV-2.*

1. INTRODUCTION

The COVID-19 diagnosis is largely determined by the results of laboratory examinations, especially biomolecular examinations [1]. At the same time, the biomolecular examination has not been widely carried out yet in Indonesia during the earlier days of the COVID-19 pandemic. The number of biomolecular laboratories in Indonesia at the beginning of the pandemic was only 12 laboratories [2]. The scarcity of biomolecular laboratories was accompanied by the scarcity of reagents. Various available reagents for the examination of SARS-CoV-2 had not obtained sufficient performance investigations yet to be circulated and used in the diagnostic process. Reagents that existed and circulated at the beginning of the COVID-19 pandemic in Indonesia were allowed to use under the Emergency Use Authorization (EUA). Furthermore, the examination of the performance of diagnostic reagents still needs to be done [2].

One kind of biomolecular examination method that is commonly used is Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The implementation of RT-PCR examinations during the early days of the COVID-19 pandemic in Indonesia often experienced a problem in terms of the availability of reagents. The fluctuation in the number of cases that sometimes sharply increased, makes it difficult to determine the number of reagents that must be provided by the laboratory. Laboratory was suggested to use identical reagents in the operation, but the sudden increment of COVID-19 cases forced the

Copyright © 2022 The Authors. Published by Atlantis Press International B.V.
This is an open access article distributed under the CC BY-NC 4.0 license -http://creativecommons.org/licenses/by-nc/4.0/.

laboratory to incorporate the use of alternative reagents, to continue the service. Moreover, the changes in reagents used by the laboratory impacted many aspects. There were changes in work procedures, monitoring, evaluation, and interpretation of the results. The use of new reagents required an optimization process followed by a continuing verification process in the laboratory to be able to determine the performance of the test and to assure the quality of the results.

Authorization for the utilization of reagents by laboratories in the COVID-19 pandemic era is very different from the conditions before the pandemic [3]. The use of diagnostic reagents in pre-pandemic conditions require several stages of validation. The initial stage is in the form of analytical validation which includes analytical sensitivity and specificity examination, which will then be followed by an examination of a large number of patient samples to determine clinical sensitivity and specificity [4]. Both stages of the examination need to be followed by registration and authorization by the competent authority, to declare that the reagents are suitable for diagnostic tests. The capacity of laboratory tests that need to be improved, as well as limitations in the provision of reference methods and the lack of explanation of clinical manifestations for determining the diagnostic criteria for this newly emerging disease, make the reagent verification carried out within limitations and issued as an Emergency Use Authorization (EUA) by the assessed agency. Authorization for emergency use will end when the Public Health Emergency (PHE) has been declared over [2], [3].

The method used to detect SARS-CoV-2 is the nucleic acid amplification test (NAAT). One of the most favorable NAAT and commonly used methods in Indonesia is not different from the one previously mentioned, which is the RT-PCR method [5]. The reverse transcriptase-polymerase chain reaction examination for SARS-CoV-2 examines the RNA of the virus through a series of processes in a cyclical manner. The method employs reverse transcriptase enzymes, polymerase enzymes, nucleotide addition, and fluorescence probes [5], [6]. This method targeted the genes that are expected to be specific for the SARS-CoV-2 virus. One of those genes is the Orf1 ab gene [6]. Orf 1 ab is one of the open reading frames that encode polypeptides, including 16 types of non-structural proteins. Most of the nucleotides in the SARS-CoV-2 virus are located in the Orf 1 a and Orf 1 ab genes, some are in the genes that encode the structural proteins [7], [8]. The aim of this study is to investigate whether the results produced by three different RT-PCR reagents which targeted Orf 1 ab genes in the examination are interchangeable.

2. MATERIALS AND METHODS

2.1. Materials

This research has been approved by the ethical committee and the Ethical Clearance Number is 074/M/KEPK/2021. Twenty-eight nasopharyngeal and oropharyngeal swab specimens in Virus Transport Media (VTM) were used in this study. Sample numbers were acquired by the calculation for comparative study with a 95% level of confidence. The samples were originated from individuals that categorized as having close contacts to confirm COVID-19 patients. These individuals were participants of the contact tracing program, initiated by the community health center (*Puskesmas*) in the Cimahi region. *Puskesmas* officials performed the samples collection and the samples were sent to the Biomolecular Laboratory of Politeknik Kesehatan Kementerian Kesehatan Republik Indonesia (Poltekkes Kemenkes RI) Bandung, for the examination. The samples collection and examination were performed in early November 2020.

The participant's identity was registered by assigned administrators to National All Record, which reports the national COVID-19 incidents. These administrators obscured the participants' identities by code. Moreover, the administrators are the only people who held the key to the code, even the investigators could not access the original data of identity of the samples source. Furthermore, the results of the examination were reported to the National All Record by the administrators. The samples were examined using the first RNA extraction and RT- PCR reagents. The detected Orf1ab gene was randomized and chosen to be included in this study, and the samples were excluded if the volume of the samples were not adequate to be processed in subsequent RNA extraction and RT- PCR analysis.

Three reagents were examined in this study. Two reagents were produced in the same country (China), one reagent is from another country (South Korea). The first reagent is Maccura® The second reagent is Biosensor® and the third reagent is Zybio®. The PCR reagents consist primarily of enzymes and reaction solutions. Enzymes in the reagents are reverse transcriptase, Taq-polymerase, and uracil N-glycosylase. The reaction solution consists of dNTPs, Mg_2^{++}, primer, and probes.

The reagents used in this study were authorized to use by WHO or by USA FDA, and the examination result was allowed to be used in patients' diagnosis and clinical decisions. The reagents possess features that differ from each other, and the difference had been translated into different protocols according to the manufacturers. Table

1 explains the feature difference among reagents used in this study.

Table 1. The feature difference among reagents used in the study [9], [11].

Features	Reagent 1	Reagent 2	Reagent 3
RNA extracts volume to total volume ratio	1:2	1:3	1:2
RNA extracts volume	20 µL	10 µL	10 µL
Reaction solution mix volume	17 µL	14 µL	8 µL
Enzyme mix volume	3 µL	6 µL	2 µL
Fluorophores required	FAM	FAM	ROX

2.2. Methods

2.2.1. RNA Extraction Methods

The RNA from the samples was extracted manually by the validated instruction by the manufacturers. Each sample was divided into three portions and included in the RNA extraction process using the first reagent, second reagent, and third reagent. Negative controls, positive controls, and internal controls were extracted along with the samples. The extraction process consists of working solution making, lysate making, the washing process, and the elution process. Extracts were separated into tubes and labeled.

2.2.2. Real-Time Reverse Transcriptase PCR Analysis

The PCR reaction mixture was prepared according to manufactures protocol. The first reagent requires a total volume of 40 µL which consists of 20 µL extract of the specimen and 20 µL PCR reagent mix. The second reagent requires a total volume of 30 µL which consists of 10 µL extract of the specimen and 20 µL PCR reagent. The third reagent requires a total volume of 20 µL which consists of 10µL extract of the specimen and 10 µL PCR reagent mix. RNA amplification was conducted with PCR Gentier 960 instrument and protocol. The PCR cycle consists of reverse transcription at 50 °C for 15 min, pre-denaturation at 95 °C for 2 min, denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 35 s, and followed by cooling at 40 °C for 10 s.

Fluorescence signal development was visualized in a real-time manner. The threshold level was adjusted to be higher than fluorescence background and negative control. The cycle threshold (CT) value was determined at the point where the fluorescence signal was leaping up higher than the threshold and started to configure a smooth sigmoidal curve.

3. RESULTS AND DISCUSSION

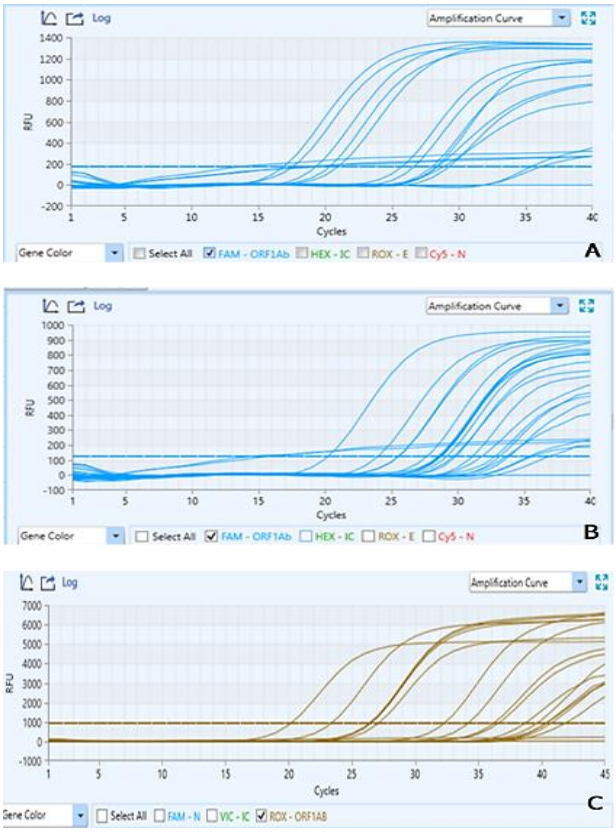

Figure 1 The amplification curve was produced by reagent 1 (A), reagent 2 (B), and reagent 3 (C).

Figure 1 shows the sigmoid curve during the real-time analysis of the RNA amplification from 28 samples with the first reagent, second reagent, and third reagent. The comparison of the CT values of the Orf 1 ab gene with the three reagents was analyzed using the Friedman test. The Differences of Orf 1 ab gene's CT values were detected (P< 0,05). Post Hoc analysis performed with the Wilcoxon test showed that the Orf 1 ab gene's CT value produced by using the first reagent was different from those produced by using the second and third reagents. The Orf 1 ab gene's CT value produced by using the second reagent was different from those produced by using the third reagent. Table 2 shows the Orf1ab gene's CT values that were produced during examination with the three reagents.

The Differences in the CT value of the Orf 1 ab gene produced by three different reagents indicate that the use

of the reagents for the diagnosis and follow-up examination of COVID-19 patients is not interchangeable. Patients that have been diagnosed with the first reagent should be evaluated in the follow-up examination with the same reagent, and the same treatment should be done also for the second and the third reagents. This is because the use of different reagents for diagnosis and the followed examination could lead to a misleading interpretation of the CT value.

Table 2. Cycle threshold numbers of the Orf 1 ab gene from examination using the three reagents.

Reagents	Range of Orf 1 ab gene CT Values	Median of Orf 1 ab gene CT Values	P-value
Reagent 1 Maccura ®	16,96 – 32,21	25,96	P<0,05
Reagent 2 Biosensor ®	12,54 – 34,29	24,87	
Reagent 3 Zybio ®	19,57 – 39,43	28-39	

Friedman test, P value in post hoc Wilcoxon: Examination with reagent 1 vs Examination with reagent 2 < 0.05; Examination with reagent 1 vs. Examination with reagent 3 < 0.05; Examination with reagent 2 vs. Examination with reagent 3 < 0.05.

Differences in the CT value of the Orf 1 ab gene on examination with different reagents, as shown by this study were also found in other studies in Korea and Austria [12], [13]. Sung et al (2020) [12] investigated the results of examinations from 118 laboratories that registered in the External Quality program Assessment (EQAS) for the examination of SARS-CoV-2 in South Korea, and successfully found some interesting findings. The EQAS program sent a set of synthetic SARS-CoV-2 samples to be examined in each laboratory. There are five groups of laboratories based on the reagents that are used to examine the Orf 1 ab gene. The group that used the first of the five reagents consisted of 68 laboratories, and in this group, the standard deviation range for the mean of Orf 1 ab gene CT values ranged from 0.76 to 1.73. The examination results in the other groups that used the same reagents also showed the same thing, which is there was a difference in the CT value of the Orf1ab gene. This finding indicates that in the laboratory group using the same reagent, variations in the CT value of the Orf 1 ab gene occurred. The variations in the CT value of the Orf 1 ab gene are also seen between one group and another. Sung's research shows that the PCR test reagent is not the sole factor that determines the CT value of the Orf 1 ab gene [12]. Other factors such as laboratory conditions, human resources as operators, and equipment could affect the results of the examination. Noting that variations in the CT value may occur in examinations with different systems, it can be stated that the use of SARS-CoV-2 RT- PCR assay for patients' follow-up should be carried out in the same system as the initial examination [13]. This study is different from Sung's finding, and also different from the study in Austria, because this study was carried out under the same laboratory conditions, human resources, and equipment. So, the factors suspected to be the ones that caused the difference in the CT value of the Orf 1 ab gene were the reagents and protocols from the manufacturers.

The CT value also correlates with the amount of genetic material (RNA) of the SARS-CoV-2 virus found in the examined sample [7]. A low CT value represents more genetic material found on examination and vice versa. The amount of genetic material in the test sample is closely related to the pre-analytical process that occurs during the sampling, evacuation of genetic material from the swab into the VTM, and transportation of the sample to the laboratory.

Various studies related to the clinical features of COVID-19 patients show that the CT value is not always in line with the severity of the clinical symptoms experienced by the patient. [8], [14], [15]. This study shows the same thing. The individuals in this study were individuals included in the contact tracing program and most of them did not show clinical symptoms of COVID-19 at the time of sampling. The CT value of the Orf 1 ab gene examined with the three different reagents in this study ranged from 12.54 - 39.43 which indicates a very wide range of Orf1 ab gene CT values among individuals with a close contact history with confirmed COVID-19 patients. Taking these research studies' results into account, it can be stated that the use of CT values for clinical decision-making for patients should still pay considerable attention to the clinical conditions and the ongoing epidemiological situation in the area. [3], [5], [15], [16].

ACKNOWLEDGMENTS

Authors would like to acknowledge Cimahi Public Health Office (*Dinas Kesehatan Kota Cimahi*), Dean of UNJANI Medical Faculty and Head of Medical Laboratory Technic Major from Bandung Health Polytechnic for allowing the investigators to volunteering in the laboratory service for the people of Cimahi and to conduct the research. We would like to thank our colleagues for their assistance and discussions during the research.

REFERENCES

[1] WHO, Laboratory testing for coronavirus disease 2019 (COVID-19) in suspected human cases, World Health Organization, 2020.

[2] Emergency Use Listing (EUL) Weekly Update : Update on submission of applications to the WHO EUL for SARS-CoV-2 Virus IVDs Summary of activities: 07 April 2020 [press release], WHO2020.

[3] S.L. Mitchell, K.S. George, D.D. Rhoads, S.M. Butler-Wu, V. Dharmarha, P. McNult, et al., Understanding, verifying, and implementing emergency use authorization molecular diagnostics for the detection of SARS-CoV-2 RNA, J Clin Microbiol, 2020. DOI : https://doi.org/10.1128/JCM.00796-20.

[4] L. Jennings, V.M.V. Deerlin, M.L. Gulley, Recommended principles and practices for validating clinical molecular pathology tests, vol. 133, Arch Pathol Lab Med, 2009, pp. 743-755. DOI: 10.5858/133.5.743.

[5] C.Y. Yu, K.G. Chan, C.Y. Yean, G.Y. Ang, Nucleic acid-based diagnostic tests for the detection SARS-CoV-2: an update, vol. 11, Diagnostics, 2021, pp. 53.DOI:https://doi.org/10.3390/diagnostics11010053

[6] D. Kalita, S. Deka, Effectiveness of different gene-target strategies for SARS-CoV-2 screening by RT-PCR and other modalities: a scoping review, vol. 9, J Med Diagn Meth, 2020, pp. 298. DOI: 10.35248/2168-9784.2020.9.298

[7] F.K. Yoshimoto, The proteins of severe acute respiratory syndrome coronavirus-2 (SARS CoV-2 or n-COV19), the cause of COVID-19, vol. 39, Protein J, 2020, pp. 198-216. DOI: 10.1007/s10930-020-09901-4

[8] ViPR, Coronaviridae gene/protein details: viprbrc.org, 2021 [cited 2021 03 September], Available from: https://www.viprbrc.org/brc/viprDetails.spg?ncbiProteinId=ABI96956&decorator=corona.

[9] Maccura, SARS COV 2 fluorescent PCR kit instruction for use, in: Ltd MBC, editor. Rev. 3 ed, Chengdu: Maccura Biotechnology Co. Ltd, 2020.

[10] Biosensor S. STANDARD M nCoV real-time detection kit instruction for use, in: SD BIOSENSOR I, editor, Gyeonggi-do: SD BIOSENSOR, 2020.

[11] Zybio, SARS COV 2 nucleic acid detection kit instruction for use, in: Zybio.inc, editor, Chongqing: Zybio.inc, 2020.

[12] H. Sung, M.G. Han, C.K. Yoo, S.W. Lee, Y.S. Chung, J.S. Park, et al., Nationwide external quality assessment of SARS-CoV-2 molecular testing, vol. 26, Emerging Infectious Diseases, South Korea, 2020, pp. 2353-2360. DOI: https://dx.doi.org/10.3201/eid2610.202551.s

[13] V. Matheeussen, V.M. Corman, O.D. Mantke, E. McCulloch, C. Lammens, H. Goossens, et al., International external quality assessment for SARSCoV-2 molecular detection and survey on clinical laboratory preparedness during the COVID-19 pandemic, vol. 25, Euro Surveill, 2020. DOI: https://doi.org/10.2807/15607917.ES.2020.25.27.2001223

[14] Keputusan Menteri Kesehatan Republik Indonesia Nomor Hk.01.07/Menkes/182/2020 Tentang Jejaring Laboratorium Pemeriksaan Coronavirus Disease 2019 (COVID-19) (2020).

[15] A.J. Kucharski, P. Klepac, A.J.K. Conlan, S.M. Kissler, M.L. Tang, H. Fry, Effectiveness of isolation, testing, contact tracing, and physical distancing on reducing transmission of SARS-CoV-2 in different settings: a mathematical modelling study, vol. 20, The Lancet Infectious Disease, 2020, pp. 1151-1160. DOI: https://doi.org/10.1016/S1473-3099(20)30457-6

[16] A. Hannecart, A. Litzroth, B. Kabamba, C. Martin, C. Wyndham-Thomas, E. Padalko, et al., Interpretation of PCR results and infectivity, Sciensano, 2020.