Application of Freeze-thaw Harvest for SARS-CoV-2 PCR EQA Panel Material

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Abstract: BACKGROUND: Currently there are more than 700 testing laboratories for COVID-19 in Indonesia. To ensure that the laboratory has a good performance, a proficiency test panel for external quality assurance program was conducted. The production of panels derived from virus isolate from cell culture, generally does not contain ribonuclease protein (RNP) as in clinical samples. OBJECTIVE: To generate a panel that resembles clinical samples, we conducted experiments to produce panels containing RNP by freeze-thaw protocol. METHODS: SARS-CoV-2 virus cultures were performed in the NIHRD BSL-3 laboratory facility. Harvesting is carried out on the 3rd, 6th, and 9th days with: no freeze-thaw process, 1 freeze-thaw process, 2 freeze-thaw processes, and 3 freeze-thaw processes. RESULTS: On the 3rd day of observation, the Ct isolates had reached an average of 11.53 and did not increase with the increase in incubation time. Meanwhile, the viral Ct became smaller in the presence of freeze-thaw treatment. RNP began to be detected on day 3 with an average Ct of 35.40 and improving with the addition of days and the number of freeze-thaw treatments. CONCLUSION: Freeze-thaw treatment can be used to improve the value of Ct however, the detected Ct RNP value was still higher than the viral Ct.

1 INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first discovered in 2019 in Wuhan and caused an outbreak of pneumonia (Zhu, Zhang, et al. 2020, Chan et al. 2020, WHO 2020b). This virus spreads and causes a pandemic. SARS-CoV-2 was first detected in Indonesia in March 2020 and at that time the laboratory designated as a testing laboratory was only the Virology laboratory in the Center for Research and Development for Biomedical and Basic Health Technology (CRDBBHT), based on Health Ministry Decree No. 658 concerning referral laboratory networks for emerging and re-emerging diseases (Health 2009).

Indonesia has a fairly high population density and is spread across 5 major islands with various transportation complexities. This of course affects the speed of sending specimens to the Virology laboratory CRDBBHT, which may cause delays in diagnosis. For this reason, the government has appointed government and private laboratories to carry out laboratory tests of SARS-CoV-2 detection in Indonesia using the real-time RT-PCR method. Additionally, there are many reagents on the market and have not been fully validated (WHO 2020a). Apart from that, only a small proportion of the laboratories that are designated as examination laboratories have the ability to carry out examinations using the PCR method. To ensure the accuracy of the final results issued by the laboratory, quality control is needed so that the results issued are accurate and reliable. One of the elements needed in quality control is external quality assurance (EQA) ((ASLM) 2020).

Materials used for testing the quality of COVID-19 laboratory may come from clinical samples or isolates obtained from viral cultures. SARS-CoV-2 has been successfully cultured and propagated using cell culture, using both primary human airway epithelial cells (AEC) and Vero African green monkey kidney epithelial E6 cells (Barrow et al. 2021). Viral growth can be seen with the emergence of damage to cells known as the cytopathic effect (CPE). Most of the viruses leave the cell and are found in the medium, but some viruses bind to the cells. To excrete cell-bound viruses, a combination of

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freeze-thaw, sonication, or a combination of both is usually used (Hierholzer, Killington, and Stokes 1996, Laposova, Oveckova, and Tomaskova 2017). Another way to get a high enough viral titer is by performing passages (Mengesha et al. 2014).

One of the internal controls used to validate the results of the PCR examination is the appearance of an RNP curve which indicates the presence of the rnp gene from human epithelial cells in the specimen being examined. Rnp is a protein found in the nucleus and cytoplasm. Some of the commercial panels used for COVID-19 EQA do not contain RNP, so some laboratories have concluded that the panels examined were invalid. The invalid status referred to here is an invalid sampling process, not the results of the examination.

In the implementation of the proficiency test organized by the Ministry of Health through the National Institute of Health Research and Development (NIHRD) in collaboration with WHO, many laboratory participants encountered difficulties in determining the test results. This is because the RNP Ct did not appear from the panel test and caused many laboratories did not to obtain a perfect result in the test. To improve the performance of the COVID-19 network laboratory in Indonesia, we are trying to produce panels containing the rnp gene so that they resemble clinical samples.

2 METHODS AND MATERIALS

In this study, we inoculated 3 SARS-CoV-2 isolates, which are preserved biological materials belonging to the national reference laboratory for COVID-19 testing under the ethical approval number LB.02.01/2/KE.432/2020 from the NIHRD ethical committee. Isolation was carried out with differences in incubation time and the amount of freeze-thawed process to see the differences of viral Ct and RNP.

2.1 Cell Line Preparation

Vero E6 cells used for virus isolation and propagation were maintained using Dulbecco's modified Eagle medium (DMEM) with the addition of 10% fetal bovine serum (FBS), l-glutamine 2 mM, gentamicin, 100 units of Penicillin, and 100 µg / mL of Streptomycin. Cells were grown in a BSL-2 laboratory using TC Flask 25cm^2 and incubated in an incubator at 37ºC and 5% CO2 for 1-2 days until it reaches 90% confluent.

2.2 SARS-CoV-2 Virus Inoculation

The SARS-CoV-2 cultures were conducted in the BSL-3 facility. When the cells were 90% monolayer, the medium in TC Flask 25cm^2 was discarded and washed with 3 mL of PBS 2 times and then added 200 µL of SARS-CoV-2 isolate. The cultured specimens were then incubated in an incubator at 37ºC and 5% CO_2 for 60 minutes. Then added with the viral culture medium used containing DMEM with 2% fetal bovine serum (FBS), 2 mM l-glutamine, gentamicin, 100 units of Penicillin, and 100 ug / mL of Streptomycin were added to the TC flask. The flasks were incubated in an incubator at 37ºC and 5% CO2 for 0, 3, 6, and 9 days.

2.3 RNP Detection

A total of 500 µL of isolates were taken from each flask and put in a micro-centrifuge tube as samples without freeze-thaw treatment. The TC flask was put into the -70oC deep freezer until frozen (1st freeze), then removed from the freezer and allowed to run until it is liquid (1st thaw). Furthermore, as many as 500 µl isolates were taken and transferred into a microcentrifuge tube as a sample for one freeze-thaw treatment. The flasks were placed in the deep freezer again until the culture freeze for the 2nd time and removed from the freezer to get the 2nd thaw. We repeat the steps to obtain 3 freeze-thaw cycles.

Isolates were extracted manually by the spin column method using the QIAamp Viral RNA Mini Kit (Qiagen) and the rnp gene was carried out using a Biorad CFX96 with a set of primer forward (AGA TTT GGA CCT GCG AGC G) reverse (GAG CGG CTG TCT CCA CAA GT) and probe (TTC TGA CCT GAA GGC TCT GCG CG) in the realtime RT-PCR method using Real-Q 2019-nCoV (Biosewoom) according to the manufacturer's protocol (WHO 2009).

The data obtained were then analyzed quantitatively descriptively to determine the freeze-thaw process against the Ct of viral and rnp genes.

3 RESULTS AND DISCUSSION

Based on the observation, it is known that the three SARS-CoV-2 virus isolates on the 3rd-day culture incubation have shown CPE or cell morphological damage when compared to the control. Observations using scanning electron microscopy (SEM) conducted by Zhu et al., showed the formation of plaque-like CPE that continues to expand in cells

infected with the SARS-CoV-2 virus as the incubation time increases (Zhu, Wang, et al. 2020).

The results of virus examination using the real-time RT-PCR method showed a significant increase in the number of viruses, from the initial average Ct 29.29 to 11.53. The Ct values did not differ significantly in the addition of the 6- and 9-day incubation periods, namely 12.68 and 12.11 (Figure 1). Therefore, it is known that incubation for 3 days is optimal in producing the SARS-CoV-2 virus.

The freeze-thaw treatment which is intended to break down cell tissue is known to increase the number of viruses harvested. However, in this experiment, the Ct value of the virus did not change significantly (Figure 2). This may be due to the majority of viral virions are already outside of the cell since the first observation (3 x 24 hours).

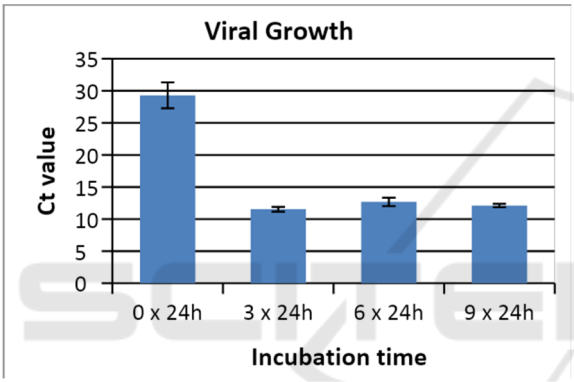

Figure 1: The viral Ct in different incubation time.

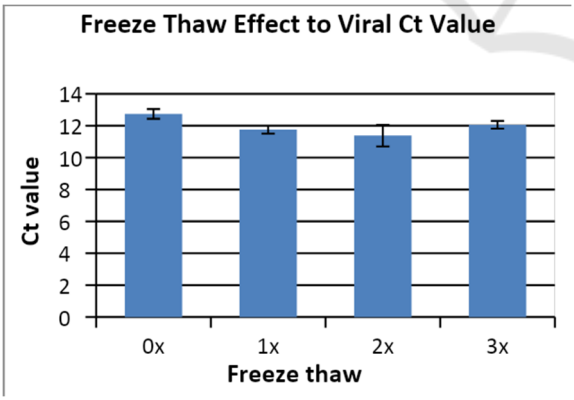

Figure 2: The viral Ct in different number of freeze thaw treatment.

Unlike the case with Ct virus, which was not significantly different from the increase in incubation time. In Figure 3 we can see that Ct for rnp has increased with increasing incubation time. Initially, even the rnp was not detected or the Ct was greater than the cut-off value. However, on the last day of observation, the average value was 22.66.

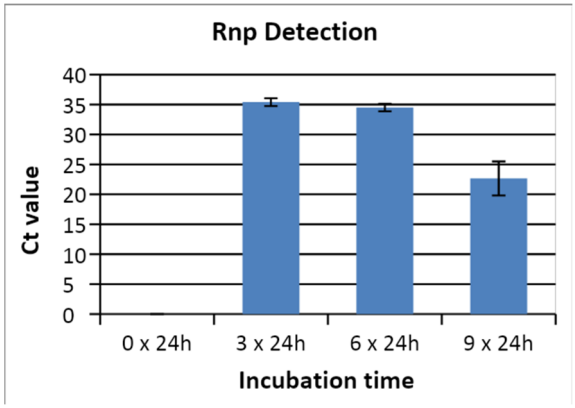

Figure 3: The RNP Ct detected in different incubation time.

In Figure 4, we can see that the Ct for rnp has improved in the presence of the freeze-thaw treatment. However, the best Ct can be obtained by 2 times freeze-thaw process, even though some studies showed that 3-5 cycles of freeze-thaw would increase the virus yield (Kong, Foster, and Foster 2008, Gupta et al. 1996).

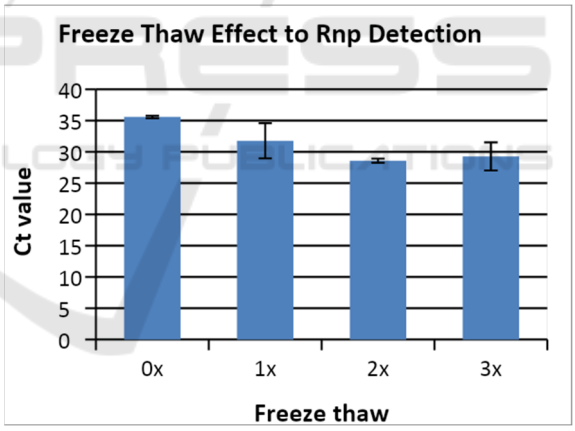

Figure 4: The rnp Ct detected in different incubation time.

The trend continues to improve for rnp detection with the addition of days. However, for the freeze-thaw treatment, it seems that the addition of freeze-thaw cycles more than 2 times did not cause a different impact up to 9 days of incubation.

The number of viruses produced was good enough on day 3 (detected Ct: 11.53). However, the number of rnp produced up to day 9 was still in the intermediate level (detected CT: 22.66). Thus, in one sample a balanced Ct condition has not been obtained. The limitation of this study is that we did not observe

the effect of incubation time and freeze-thaw process earlier than 3 days and later than 9 days of incubation.

Another study on the freeze-thaw aspect of RNA detection by PCR method was carried out on the dengue virus. The results showed that the freeze-thaw process did not affect RNA detection (Anwar et al. 2009). Therefore, this method is promising for continuous development to obtain optimal Ct. It is necessary to carry out a further study with a longer incubation time to determine the best conditions for producing a balanced Ct number between viral genes and rnp.

4 CONCLUSIONS

The process of harvesting the SARS-CoV-2 virus culture by the freeze-thaw process is sufficient for an incubation period of 3 days and can be used for producing EQA panel material. However, the detected Ct rnp value was still higher than the viral Ct. Thus, it is necessary to carry out further optimization in the process of harvesting the SARS-CoV-2 virus as a panel material for proficiency testing that resembles clinical specimens.

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REFERENCES

(ASLM), African Society for Laboratory Medicine. 2020. "Assuring quality COVID-19 test results: quality control and external quality assurance strategies."

Anwar, Azlinda, Guoqiang Wan, Kaw-Bing Chua, Joseph Thomas August, and Heng-Phon Too. 2009. "Evaluation of pre-analytical variables in the quantification of dengue virus by real-time polymerase chain reaction." *The Journal of Molecular Diagnostics* 11 (6):537-542.

Barrow, Kaitlyn A, Lucille M Rich, Elizabeth R Vanderwall, Stephen R Reeves, Jennifer A Rathe, Maria P White, and Jason S Debley. 2021. "Inactivation of Material from SARS-CoV-2-Infected Primary Airway Epithelial Cell Cultures." *Methods and Protocols* 4 (1):7.

Chan, Jasper Fuk-Woo, Shuofeng Yuan, Kin-Hang Kok, Kelvin Kai-Wang To, Hin Chu, Jin Yang, Fanfan Xing, Jieling Liu, Cyril Chik-Yan Yip, and Rosana Wing-Shan Poon. 2020. "A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster." *The lancet* 395 (10223):514-523.

Gupta, Chander Kanta, Jeanne Leszczynski, Rajesh K Gupta, and George R Siber. 1996. "Stabilization of respiratory syncytial virus (RSV) against thermal inactivation and freeze-thaw cycles for development and control of RSV vaccines and immune globulin." *Vaccine* 14 (15):1417-1420.

Health, Ministry of. 2009. "Ministry Decree The Reublic of Indonesia No. 658/MENKES/PER/VIII/ 2009 – Laboratory Network for New Emerging and Re-Emerging Disease Diagnistic.". Available from: https://pamki.or.id/download/peraturan-menteri-kesehatan-republik-indonesia-nomor-658-menkes-per-viii-2009-tentang-jejaring-laboratorium-diagnosis-penyakit-infeksi-new-emerging-dan-re-emerging/.

Hierholzer, JC, RA Killington, and A Stokes. 1996. "Preparation of antigens." In *Virology Methods Manual*, 47-70. Elsevier.

Kong, Byung-Whi, Linda K Foster, and Douglas N Foster. 2008. "A method for the rapid isolation of virus from cultured cells." *Biotechniques* 44 (1):97-99.

Laposova, Katarina, Ingrid Oveckova, and Jana Tomaskova. 2017. "A simple method for isolation of cell-associated viral particles from cell culture." *Journal of virological methods* 249:194-196.

Mengesha, AA, B Hurisa, T Tesfaye, H Lemma, D Niguse, GG Wold, A Kebede, T Mesele, and K Urga. 2014. "Adaptation of local rabies virus isolates to high growth titer and determination of pathogenicity to develop canine vaccine in Ethiopia." *J Vaccines Vaccin* 5 (245):2.

WHO. 2009. "CDC Protokol of Realtime RTPCR for Influenza A (H1N1)." accessed 10 June. https://www.who.int/csr/resources/publications/swineflu/CDC RealtimeRTPCR_SwineH1Assay-2009_20090430.pdf.

WHO. 2020a. "Molecular assays to diagnose COVID-19: Summary table of available protocols. ." https://www.who.int/publications/m/item/molecular-ssaysto-diagnose-covid-19-summary-table-of-available-protocols.

WHO. 2020b. "Pneumonia of unknown cause — China.", accessed 21 April. https://www.who.int/csr/don/05-january-2020-pneumonia-of-unkown-cause-china/ en/.

Zhu, N, W Wang, Z Liu, C Liang, W Wang, F Ye, B Huang, L Zhao, H Wang, and W Zhou. 2020. Morphogenesis and cytopathic effect of SARS-CoV-2 infection in human airway epithelial cells. Nat Commun 11: 3910.

Zhu, Na, Dingyu Zhang, Wenling Wang, Xingwang Li, Bo Yang, Jingdong Song, Xiang Zhao, Baoying Huang, Weifeng Shi, and Roujian Lu. 2020. "A novel coronavirus from patients with pneumonia in China, 2019." *New England journal of medicine*.