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OPTIMIZATION OF DENATURATION TEMPERATURE AND TIME USING REAL-TIME PCR METHOD IN HEPATITIS B TEST

OPTIMASI SUHU DAN WAKTU DENATURASI MENGGUNAKAN METODE REAL-TIME PCR DALAM PEMERIKSAAN HEPATITIS B

Original Research Article
Penelitian

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A B S T R A C T

Background: *The hepatitis B test can be conducted using the real-time PCR method. In chronic cases, the test is performed by detecting one of the Hepatitis B Virus (HBV) specific genes, HBcAg. The real-time PCR method requires optimization to obtain optimal results.* ***Purpose:*** *This research aims to determine the optimal temperature and time for denaturation in the hepatitis B test using the real-time PCR method.* ***Method:*** *The research method used was a quasi-experiment involving variations in temperature (94 °C, 95 °C, and 96 °C) and time (10 seconds, 15 seconds, and 20 seconds) for denaturation. Data processing resulted in static group comparisons based on 27 primary data from the Cycle Threshold (CT) value.* ***Result:*** *Variations in temperature conditions, specifically at 94 °C, 95 °C, and 96 °C, combined with a denaturation time of 10 seconds, yielded the mean CT values of 26.495, 26.355, and 26.003, respectively. When the temperature conditions were maintained at 94°C, 95°C, and 96°C, with a denaturation time of 15 seconds, yielded the mean CT values of 25.962, 25.641, and 25.396. Similarly, under temperature conditions of 94 °C, 95 °C, and 96 °C with a denaturation time of 20 seconds, yielded the mean CT values of 26.544, 26.505, and 25.830 were obtained.* ***Conclusion:*** *Optimal results in this research are obtained through the acquisition of the smallest CT value, namely at temperature conditions of 96 °C and 15 seconds.*

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A B S T R A K

Latar belakang: Pemeriksaan hepatitis B dapat dilakukan dengan metode *real-time* PCR. Pada kasus kronis, pemeriksaan dilakukan dengan mendeteksi salah satu gen spesifik *Hepatitis B Virus* (HBV) yaitu HBcAg. Pemeriksaan dengan metode *real-time* PCR memerlukan optimasi untuk mendapatkan hasil yang optimal. **Tujuan:** Penelitian ini bertujuan untuk menentukan suhu dan waktu denaturasi yang optimal untuk pemeriksaan hepatitis B menggunakan metode *real-time* PCR. **Metode:** Metode penelitian yang digunakan adalah eksperimen semu dengan variasi suhu (94°C, 95°C, dan 96°C) dan waktu (10 detik, 15 detik, dan 20 detik) untuk denaturasi. Hasil pengolahan data berupa perbandingan kelompok statis berdasarkan 27 data primer dari nilai *Cycle Threshold* (CT). **Hasil:** Kondisi suhu 94 °C, 95 °C, dan 96 °C dengan waktu denaturasi 10 detik menghasilkan nilai CT masing-masing sebesar 26,495; 26,355; dan 26,003. Pada kondisi suhu 94 °C, 95 °C, dan 96 °C dengan waktu denaturasi 15 detik memperoleh nilai CT rata-rata sebesar 25,962; 25,641; dan 25,396. Demikian pula pada kondisi suhu 94 °C, 95 °C, dan 96 °C dengan waktu denaturasi 20 detik, diperoleh nilai CT rata-rata sebesar 26,544; 26,505; dan 25,830. **Kesimpulan:** Hasil optimal pada penelitian ini didapatkan dari perolehan nilai CT yang paling kecil yaitu pada kondisi suhu 96 °C dan 15 detik.

Kata kunci:
Denaturasi, HBcAg, Hepatitis B, PCR, *Real-time*

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INTRODUCTION

Hepatitis B is a liver disease that can be transmitted through contact with blood or body fluids. The disease can be categorized as acute or chronic, depending on the time of infection and incubation (Soedarto, 2019). If left untreated, prolonged hepatitis B infection can lead to liver cirrhosis (Malau and Wicaksono, 2021) or even primary hepatic carcinoma (liver cancer) (Sastrawinata, 2008). Indonesia has 28 million cases of hepatitis B, with 14 million being chronic and 1.4 million carrying the risk of liver cancer (Ministry of Health, 2021) due to the large number of asymptomatic cases in Indonesia (Wijayanti, 2016), treatment is often delayed, leading to chronic cases. In response, the government has issued regulations such as Ministry of Health Regulation Number 52 of 2017, aiming to eliminate mother-to-child transmission of HIV, syphilis, and hepatitis B, and Ministry of Health Regulation Number 53 of 2015, focusing on the management of viral hepatitis.

Infection with the *Hepatitis B Virus* (HBV) causes this infectious disease. HBV belongs to the *Hepadnavirus* family under the genus *Orthohepadnavirus* (ICTV, 2021). *Hepatitis B surface Antigen* (HBsAg) encases the outside of the virus with a lipid envelope. The virus contains an icosahedral nucleocapsid comprising of *Hepatitis B e Antigen* (HBeAg) and *Hepatitis B core Antigen* (HBcAg) proteins (Irianto, 2014). These antigen proteins are utilized as markers to detect the presence of HBV in a sample. Various methods can be employed to detect HBV, including the checking of *Serum Glutamic Oxaloacetic Transaminase* (SGOT) and *Serum Glutamic Pyruvic Transaminase* (SGPT) levels in the blood, using serological and molecular-based methods (Ghosh *et al*., 2015).

High levels of SGOT and SGPT can indicate the need for hepatitis B testing because their concentrations can increase due to inflammation from liver cirrhosis (Rosida, 2016). However, increased levels of these two enzymes are not entirely specific, as they can also increase in response to other types of tissue damage (Thapa and Walia, 2007). SGOT can increase due to damage in the heart, skeletal muscle, kidney, brain, pancreas, spleen, and lung, while SGPT can increase due to damage in the heart, muscle, and kidney (Rosida, 2016).

There are times when all HBsAg has been neutralized by anti-HBs and neither HBsAg nor anti-HBs is detectable, this period is called the window period. IgM anti-HBc is the only serologic marker of infection during this period (Long *et al*., 2012). This situation could lead to false-negative outcomes that could impact the patient's diagnosis and treatment. Consequently, conducting a real-time PCR test for hepatitis B is necessary due to its high level of sensitivity and specificity as well as its broad detection capabilities.

The real-time PCR process is a repetitive cycle that includes denaturation, annealing, and extension by the DNA polymerase enzyme (Fatchiyah *et al*., 2018). The temperature used for annealing must be optimum to prevent failure of the real-time PCR process (Amanda, 2020). Temperature conditions that are too low will result in mispriming, and temperatures that are too high can cause the primer not to stick because it reaches the *Melting Temperature* (TM) (Rybicki, 2014). This TM determination is based on the calculation of primer and template DNA binding (Fatchiyah *et al*., 2018). The temperature range usually used for this process is between 50 - 60 °C, while the time required for this process is usually 15 - 60 seconds (Yusuf, 2010). Denaturation is also crucial because it breaks the double chain on the DNA template into a single chain. The denaturation stage can be influenced by temperature and time. In the process, the denaturation stage of real-time PCR is carried out by heating at 90 - 95 °C (Sadewa, 2022). Denaturation temperatures that are too low can cause the double strand on the DNA template to not break properly, while temperatures that are too high will cause reduced enzyme activity. Meanwhile, if the time used is too fast, the process of breaking the nucleic acid chain will not be complete. A denaturation time of more than 3 minutes will also cause inactivity of DNA polymerase (Lorenz, 2012). Therefore, to obtain representative results, it is necessary to optimize the denaturation process, by optimizing either the temperature or the time.

Hepatitis B testing with real-time PCR can be performed by detecting complete virions (HBV DNA/RNA, HBcAg, HBeAg, and HBsAg), empty viral particles (HBsAg and HBcAg without HBV DNA/RNA), viral particles containing HBV DNA/RNA, and HBcAg (Luckenbaugh *et al*., 2015). In the serum of patients with chronic hepatitis B, HBcAg can be detected where the presence of the gene can indicate viral replication activity (Monjezi *et al*., 2013) and its quantity correlates with HBV DNA (Kimura, *et al*., 2003). Therefore, the detection of the HBcAg gene can also be a support for diagnosis or to determine therapy in hepatitis B patients (Raihan *et al*., 2015).

This research used a quasi-experimental design, optimizing HBV detection using the real-time PCR method. The focus of optimization in this research was on the denaturation stage, specifically involving temperature and time. The temperature variations used were 94 °C, 95 °C, and 96 °C, while the time variations included 10 seconds, 15 seconds, and 20 seconds. The experimental design adopted in this research is a static group comparison, with data collected based on the *Cycle Threshold* (CT) value in HBV detection by the real-time PCR method.

MATERIAL AND METHOD

Molecular testing in this research was conducted using real-time PCR, which principally detects the coding genes of specific *Hepatitis B Virus* (HBV) antigens in real-time (as the cycle progresses). The primers used in this research were the result of in silico design which was used as a medium for hepatitis B virus detection

by real-time PCR method (Merdekawati and Nurhayati, 2023). The sequence of the forward and reverse primers is presented in Table 1.

The research unit used serum from patients confirmed positive for chronic hepatitis B from UPTD Puskesmas Sukamulya, Kuningan Regency. This research was conducted at the Molecular Biology Laboratory of Medical Laboratory Technology at the Health Polytechnic of the Ministry of Health Bandung from January 2023 to May 2023. This research has been approved for publication with the issuance of ethical approval statement No. 59/KEPK/EC/V/2023. The review was conducted by the *Health Research Ethics Commission* (KEPK) of the Directorate of Health Polytechnic of the Ministry of Health Bandung.

Table 1. Data on forward and reverse primers used

Sequence primer	PF 5'AACATTGTTCACCTCACCAT'3 PR 5'TCCCGAGATTGAGATCTTCT'3
Product size	403
Length	20
Melting Temperature (TM)	55.14
GC%	45.00
ΔG kcal/mol	1.17
TM (°C)	37.7

PF = Primer forward and PR = Primer reverse

Sample extraction

200 µL of serum sample and 400 µL of VB lysis buffer were added to the microcentrifuge tube. The mixture was vortexed and incubated for 10 minutes at room temperature (25 °C). Next, 450 µL of AD buffer was added to the tube and then vortexed. The VB column was placed into the collection tube, and 600 µL of lysate from the microcentrifuge was transferred into the VB column. It was then centrifuged at 4 °C at 15.000 xg for 1 minute. The remaining liquid in the collection tube was discarded, then the VB column was put back in the same collection tube. Then the remaining lysate in the microcentrifuge was transferred into the VB column and centrifuged again at 4°C at 15.000 xg for 1 minute. The collection tube was discarded and replaced with a new one. Following this, 400 µL of W1 buffer was added to the VB column and centrifuged at 4 °C at 15.000 xg for 30 seconds. The remaining liquid in the collection tube was discarded, and the VB column back was put back in the same collection tube. Then 600 µL wash buffer was added into the VB column, then centrifuged again at 4 °C at 15.000 xg for 30 seconds, and the liquid in the collection tube was discarded. After that, it was centrifuged again at 4 °C at 15.000 xg for 3 minutes to dry the VB column. The dried VB column was then placed into a new microcentrifuge tube. Then 50 µL of RNase-free water was added to the white matrix in the center of the VB and it was allowed to stand for 3 minutes to ensure the RNase-free water was well absorbed into the matrix. Finally, it was centrifuged at 4 °C at 15.000 xg for 1 minute.

Preparation of master mix

The final concentration which was 4000 nM, 10 µL of GoTaq qPCR master mix, 0.8 µL of forward primer, 0.8 µL of reverse primer, 0.2 µL of CXR reference dye, and 8.2 µL of nuclease-free water were added to the aliquot. The mixture was vortexed until homogeneous, then it was spun down for 10 seconds. Subsequently, 20 µL of the master mix was pipetted into each PCR tube. Next, 3 µL of template DNA was pipetted into each PCR tube, followed by vortexing and spinning down for 10 seconds.

Amplification process

The amplification process was carried out using *Tianlong* real-time PCR. The first step involved activating the polymerase enzyme at 95 °C for 2 minutes in 1 cycle. The second stage included denaturation at 94 °C, 95 °C, and 96 °C for 10 seconds, 15 seconds, and 20 seconds in 40 cycles. The third stage was annealing which was carried out at 50 °C for 60 seconds in 40 cycles. The fourth stage was extension which was carried out at 60 °C for 60 seconds in 40 cycles. The last stage was cooling which was carried out at 35 °C for 30 seconds for 1 cycle.

RESULT

The entire amplification results, obtained from denaturation temperature variations at 94 °C, 95 °C, and 96 °C, along with denaturation time variations of 10 seconds, 15 seconds, and 20 seconds, were processed using Microsoft Excel software to generate a graph (Figure 1). In addition to the graph, the data was presented in tabular form as shown in Table 2.

Based on Figure 1, the data show that the CT value tends to decrease when the denaturation temperature is increased. At 94 °C, 95 °C, and 96 °C, the graph illustrates a decrease in the CT value from 10 seconds to 15 seconds, followed by an increase when the time is extended to 20 seconds.

Table 2. *Cycle Threshold* (CT) results of temperature and time variations in the denaturation

Temperature (°C)	Result			Mean		
	10 seconds	15 seconds	20 seconds	10 seconds	15 seconds	20 seconds
94	26.879	26.264	26.207	26.495	25.962	26.544
	26.273	25.851	26.589			
	26.332	25.771	26.837			
95	27.160	26.457	26.816	26.355	25.641	26.505
	26.752	25.355	26.785			
	25.152	25.111	25.913			
96	26.590	25.566	26.324	26.003	25.396	25.830
	25.637	24.945	25.363			
	25.782	25.678	25.804			

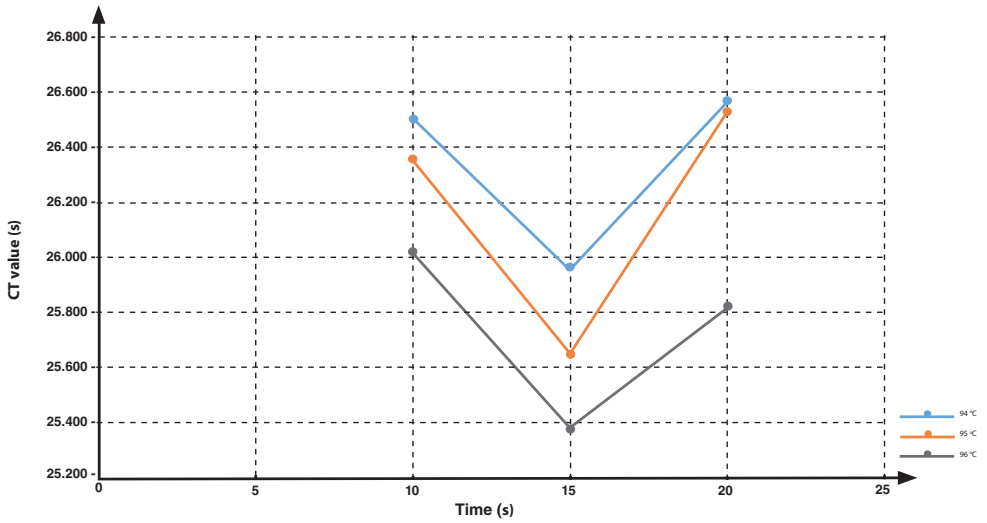

Figure 1. Real-time PCR optimization at various temperatures and times for denaturation

DISCUSSION

The detection of this gene was performed using the GoTaq qPCR Master Mix Promega kit, which detects the target gene using an intercalating agent, in this case, BRYT Green Dye with CXR as a reference dye. In the real-time PCR amplification process, the gene detected is SYBR Green, and ROX serves as the reference dye. This is due to the similarities between SYBR Green and BRYT Green, as well as between ROX and CXR (Bhakti, 2022). The similarity of SYBR Green with BRYT Green lies in their characteristics that can be read at a wavelength of 493 – 530 nm. Similar characteristics are also owned by reference dye ROX with CXR (DNA-Technology, 2021).

In this research the optimum condition is determined based on the lowest CT value. The lowest CT value means the faster the fluorescence signal reading appears to be able to cross the threshold line (Siswanto, 2019). The results of the temperature and time variations in the denaturation process carried out in this research produced 27 data, where the data with the lowest mean CT value of 25.396 was produced from 96°C temperature conditions with a denaturation time of 15 seconds. However, the CT values in the data from this research exhibit minimal differences, so the varied conditions can be said not to cause significant differences in CT values. In addition, the research data from all varied conditions are still in the same clinical range, which is in the low virus concentration group (Solange et al., 2014).

In this research, the optimal denaturation temperature was found to be 96°C, which differs from the temperature specified in the insert kit. Several possibilities cause the difference between the optimal temperature and the temperature in the insert kit. This is related to the purpose of optimization itself, which aims to find conditions that can produce the highest-quality product, which can be related to the use of available tools and materials. According to Bhakti (2022), optimization involves finding the right composition among the master mix kit, primer-probe, and real-time PCR tool, as well as determining the appropriate temperature and cycle for testing the target DNA. This

shows that maximum amplification results do not always occur under the temperature conditions listed on the kit, but rather under conditions that have been adjusted to the specific materials and equipment used. Better results can also be shown by increasing the denaturation temperature to be higher (Korner, 2017). The results of this research demonstrate that a higher temperature, specifically 96°C, resulted in CT values that tended to be lower than those at 94°C and 95°C, across denaturation times of 10 seconds, 15 seconds, and 20 seconds.

The selection of denaturation time and temperature in the *Hepatitis B Virus* (HBV) test using the real-time PCR method can also consider the effectiveness of the test time. The effectiveness of the examination time is important to analyze to determine which aspects contribute to the total time spent on the examination, so that several efforts can be reduced to shorten the examination time (Eppendorf, 2020). Modifications to shorten the examination time can be achieved by shortening the time and increasing the denaturation temperature (Thermofisher, 2023). In this research, amplification conducted with a 10-second denaturation took 2 hours 56 minutes 40 seconds, a 15-second denaturation took 3 hours, and a 20-second denaturation took 3 hours 3 minutes 20 seconds. Based on the results of CT data analysis, denaturation time of 15 seconds yielded the lowest CT value. This shows that the selection of denaturation time must be appropriate. Although it can shorten the time, the fast denaturation time may yield non-optimal CT values.

CONCLUSION

The results of this research lead us to conclude that the optimal temperature and time for denaturation in the real-time PCR method for detecting the hepatitis B virus are 96°C and 15 seconds.

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