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Diagnostic Performance of Precore Protein 22 Kilodalton Levels of HBV DNA in Chronic Hepatitis B Patients

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ABSTRACT

Hepatitis B Virus (HBV) infection causes inflammation of the liver, which has a high prevalence in both Indonesia and the world. Serum HBV deoxyribonucleic acid (DNA) is important in determining the initiation therapy for Chronic Hepatitis B (CHB) patients. However, it has several limitations. Precore protein 22 kilodalton (p22cr) is synthesized from the HBV gene in hepatocytes, representing covalently closed circle (ccc) DNA. This study aimed to analyze the diagnostic performance of p22cr levels on HBV DNA in CHB patients. An observational analytic study with a cross-sectional approach was conducted on 83 CHB patients who were examined at the Clinical Pathology Laboratory of Dr. Moewardi General Academic Hospital in December 2020. Blood plasma samples were taken for HBV DNA and p22cr examination by using Polymerase Chain Reaction (PCR) and Enzyme-Linked Immunosorbent Assay (ELISA), respectively. The cut-off level of p22cr was determined by the Receiver Operating Curve (ROC) with the widest area Under the Curve (AUC). Sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV), Positive Likelihood Ratio (PLR), Negative Likelihood Ratio (NLR), and accuracy were calculated for the diagnostic performance of p22cr. The cut-off point of p22cr on HBV DNA ≥ 20,000 IU/mL was 7.440 ng/mL with AUC 0.693 (p=0.003). The diagnostic performance of p22cr levels on HBV DNA obtained 44.44% sensitivity, 82.98% specificity, 66.67% PPV, 66.10% NPV, 2.61 PLR, 0.67 NLR, and 66.27% accuracy. P22cr level has a good specificity so it can be an alternative examination of HBV DNA in making decisions on therapy in patients with chronic hepatitis B. Further research needs to be done using HBcrAg and excluding elderly patients.

Keywords: Chronic hepatitis B, HBV DNA, p22cr

INTRODUCTION

Hepatitis B Virus (HBV) infection causes inflammation of the liver. The Hepatitis B virus is an enveloped DNA virus from the Hepadnaviridae family. Chronic infection occurs when hepatitis B surface antigen (HBsAg) is still detected for more than 6 months.[1] The process of liver parenchymal destruction in chronic HBV infection may continue, starting from the formation of fibrotic tissue, sometimes developing into cirrhosis and hepatocellular carcinoma (HCC).[2] The World Health Organization (WHO) reported that approximately 257 million people or 3.5% of the world's population suffered from chronic HBV infection in 2015.[3] The results of Riset Kesehatan Dasar (Riskesdas) in 2018 showed an increase in hepatitis cases, from 0.3% in 2013 to 0.39% in 2018 and most of all viral hepatitis cases are hepatitis B.[4,5]

Hepatitis B virus DNA level shows the presence of the Dane particle, the whole particle of HBV in the circulation, which reflects the production of active replication in hepatocytes.[6] Quantitative HBV DNA serum examination has an important function in the management of CHB patients and is recommended by WHO. It has a correlation with intrahepatic cccDNA, which is the key marker of the virus replication process. The WHO guideline from 2015 recommends that therapy should be initiated in CHB patients over 30 years of age, with elevated alanine aminotransferase (ALT) and HBV DNA levels > 20,000 IU/mL, regardless of hepatitis B envelope antigen (HBeAg).[3] However, serum HBV DNA has a lower correlation with intrahepatic cccDNA ($r = 0.664$; $p < 0.001$) at baseline than HBV ribonucleic acid (RNA) ($r = 0.781$, $p < 0.001$) and hepatitis B core-related antigen (HBcrAg) ($r = 0.741$, $p < 0.001$).[7] It also has limitations such as expensive costs, not being widely available except in larger laboratories, and requiring laboratory expertise.

Precore protein 22 kilodalton is a 22 kilodalton (kDa) molecular weight protein that is synthesized in hepatocytes when infected by HBV.[8] It is the dominant precore/core protein in HBV-DNA negative particles and the largest protein in hepatitis B core-related antigen (HBcrAg).[9] The European

Association for the Study of the Liver has proposed HBcrAg as a new biomarker for hepatitis B infection that can describe the amount and activity of cccDNA.[10]

Studies on the diagnostic performance of p22cr, which is the most dominant protein of HBcrAg, have not been conducted. This study aimed to analyze the diagnostic performance of p22cr level on HBV DNA in CHB patients.

METHODS

An observational analytic study with a cross-sectional approach was conducted at the Clinical Pathology Laboratory of Dr. Moewardi General Hospital Surakarta in September-December 2020. The research subjects were CHB patients who were diagnosed by an internist at Dr. Moewardi General Hospital of Surakarta. The inclusion criteria of this study were CHB patients aged > 18 years who have not been treated and data were obtained from medical records, HBeAg negative, performed HBV DNA testing, and agreed to participate in the study by signing informed consent conducted by the researcher. The exclusion criteria were patients with hepatocellular carcinoma confirmed by imaging or pathology examination. The number of research subjects was 83 patients, carried out by consecutive sampling and using blood plasma for the sample.[11] This study has received approval from the biomedical research ethics commission of Dr. Moewardi General Hospital of Surakarta number 1.283/XI/HREC/2020.

HBV DNA levels were assessed using the PCR method with AccuPower® HBV Quantitative PCR kit and the Bioneer Exicycler™ 96 to detect the HBV genome. The HBV DNA was classified into 2 groups, 20,000 IU/mL and < 20,000 IU/mL, based on the level of initiation therapy.[3]

P22cr level was measured using the ELISA method with MyBioSource Human P22cr ELISA kit and the Rayto RT-2100C microplate reader. Antigen-antibody reaction in ELISA will produce a color change in the sample. The optical density value was determined using a microplate reader at a wavelength of 450 nm, after calculation the p22cr results were obtained.

The platelet count was examined using an Automatic Flow cytometry hematology analyzer (XN-series, Sysmex, Kobe, Japan), while the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were examined using an Automatic chemistry analyzer (ADVIA series, Siemens, Erlangen, Germany).

All the laboratory parameters were carried out in an accredited laboratory. Internal quality control of all laboratory parameters and the Copeptin assay was performed before the study.

Statistical analysis was performed using SPSS version 23.0 for Windows. Descriptive analysis of gender was expressed in frequency and percentage, while age, platelet count, ALT, AST, HBV DNA ≥ 20,000 IU/mL, HBV DNA < 20,000 IU/mL, p22cr level ≥ cut-off, and p22cr level < cut-off were expressed as mean±Standard Deviation (SD). The cut-off of p22cr was determined by ROC. Sensitivity, specificity, PPV, NPV, PLR, NLR, and accuracy were calculated to evaluate the diagnostic performance of p22cr on HBV DNA.

RESULTS AND DISCUSSIONS

The basic characteristics of the research subjects (Table 1) showed that the mean age in this study was 48.43±13.74 years old. This result is consistent with the epidemiological research by Muljono that the distribution of positive HBsAg in Indonesia is mostly found in the age range of 45-49 years old.[12] Hepatitis B is a viral infection that can develop into a chronic form. Centers for Disease Control and Prevention states that 2-6% of people infected with HBV will develop a chronic disease.[13] Research by Ie *et al.* reported that HBV infection occurs mostly in adulthood (17-25 years old) because the risk of HBV transmissions, such as sexual activity and use of non-sterile needles, is very high at that age.[14] A person who is infected with HBV in adulthood does not realize it because he is in an asymptomatic immune tolerance phase for 10-20 years. These patients will realize that they have been infected when they are in the immune clearance phase at above 40 years old.[15]

Most of the subjects in this study were male (59%). This is consistent with previous studies, which stated that the prevalence of males with hepatitis B was higher.[16,17] Males are more susceptible to high-risk behavior for HBV transmission such as risky contact and activities that can lead to blood contact.[18] Sex hormones also play a role in CHB infection. Androgen receptors actively bind to viral enhancer I and stimulate viral transcription, whereas estrogen receptor α stops the nuclear factor hepatocytes 4α from enhancer I activation and then decreases viral transcription. Immune clearance against HBV antigen is faster in females so the development of hepatitis B disease can is easier to control.[19]

Several laboratory parameters were used to assess liver disease progressions in this study, such as platelet count, ALT, and AST. The median platelet

Table 1. Basic characteristics of the research subjects

Variable	Total (n=83, 100%)	Mean±SD[a]	Median (min-max)[b]
Age (years old)	83 (100%)	48.43±13.74	
Gender			
Male	49 (59%)		
Female	34 (41%)		
Platelet count (thousand/mL)	64 (77.1%)		255 (38-550)
Transaminase enzymes level (U/L)			
ALT	65 (78.3%)		25 (6-526)
AST	63 (75.9%)		29 (15-508)
HBV DNA (IU/mL)			
≥ 20,000	36 (43.4%)		4.45×10^5
< 20,000	47 (56.6%)	$2.9 \times 10^3 \pm 4.26 \times 10^3$	$(2.06 \times 10^4 - 5 \times 10^7)$

Abbreviations: ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; HBV: Hepatitis B Virus; DNA: Deoxyribonucleic Acid; U: unit; mL: milliliter; L: liter; SD: Standard Deviation; Min: minimum; Max: maximum.
a: normally distributed data
b: not normally distributed data

count was 255 (38-550) thousand/mL obtained from 64 (77.1%) patients. AST levels in this study had a median of 29 (15-508) U/mL obtained from 63 (75.9%) patients, while ALT levels in this study had a median of 25 (6-526) U/mL obtained from 65 (78.3%) patients. This suggests that the study subjects were in the CHB immune clearance phase. This phase is characterized by the immune system actively attacking HBV-infected hepatocytes. This results in hepatocyte injury, which triggers the inflammatory process causing an increase in ALT and AST. The immune system that succeeds in suppressing the virus will cause a significant decrease in the amount of HBV DNA followed by the normalization of ALT and AST.[15] Thrombocytopenia is common in people with advanced chronic liver disease with severe liver damage or fibrosis such as cirrhosis or HCC. Thrombocytopenia is caused by 2 main mechanisms, namely platelet sequestration in the spleen and decreased production of thrombopoietin by the liver. The cause of the sequestration of platelets in the spleen is related to congestive splenomegaly due to portal hypertension, resulting in the redistribution of platelets from the circulation pool to the splenic pool. Thrombopoietin is a hormone that regulates megakaryocytes and platelet production. The main production of thrombopoietin is in the liver, if there is severe liver damage, thrombopoietin also decreases.[20]

HBV DNA data in this study showed that 36 (43.4%) patients had HBV DNA ≥ 20,000 IU/mL with a median of 4.45×10^5 (2.06×10^4-5×10^7), which means that therapy initiation is necessary for this group according to the WHO algorithm, while 47 (56.6%) patients had HBV DNA < 20,000 IU/mL with a mean of $2.9 \times 10^3 \pm 4.26 \times 10^3$ IU/mL, which means that therapy initiation is not necessary for this group.[3]

Comparison of plasma p22cr levels with HBV DNA obtained the ROC curve (Figure 1) with AUC 0.693, p=0.003 (weak). The optimal cut-off value of p22cr levels was 7.440 ng/mL, with a sensitivity of 44.44%, and specificity of 82.98% (Table 2).

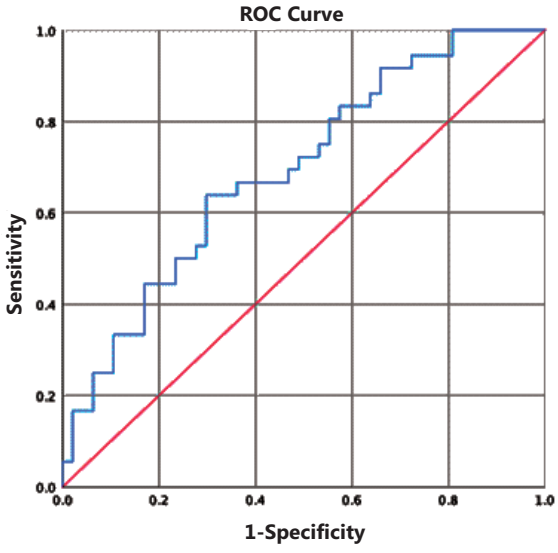

Figure 1. ROC analysis of p22cr level against HBV DNA

Table 2. Cut-off of p22cr levels based on HBV DNA levels

AUC	Sensitivity (%)	Specificity (%)	Cut-off (ng/mL)	p
0.693	44.44	82.98	7.440	0.003

Table 3. 2x2 table of diagnostic test between p22cr levels based on HBV DNA as the reference standard

Variable	HBV DNA (IU/mL)		Total
	≥ 20,000	< 20,000	
	n (%)	n (%)	n (%)
p22cr levels (ng/mL)			
≥ 7.440	16 (19.3%)	8 (9.6%)	24 (28.9%)
< 7.440	20 (24.1%)	39 (47%)	59 (71.1%)
Total	36 (43.4%)	47 (56.6%)	83 (100%)

Abbreviations: HBV: Hepatitis B Virus; DNA: Deoxyribonucleic Acid; p22cr: Precore Protein 22 Kilodalton; IU: International Unit; ng: nanogram; mL: milliliter; n: total

Examination of p22cr levels in this study showed that 24 (28.9%) patients had p22cr levels ≥ 7.440 ng/mL and 59 (71.1%) patients had p22cr levels < 7.440 ng/mL (Table 3). Sixteen of 24 patients with p22cr levels ≥ 7.440 ng/mL had HBV DNA ≥ 20,000 IU/mL, which required therapy initiation and 39 of 59 patients with p22cr levels < 7.440 ng/mL had HBV DNA < 20,000 IU/mL, which did not require therapy initiation. This shows that patients with levels ≥ 7.440 ng/mL (mean 8.747±0.670 ng/mL) are expected to have an HBV DNA ≥ 20,000 IU/mL to be qualified for therapy initiation of hepatitis B.

Table 4. Diagnostic performance p22cr levels based on HBV DNA

Diagnostic Test	p22cr Level	
Sensitivity (95% CI)	44.44%	(27.94%-61.90%)
Specificity (95% CI)	82.98%	(69.19%-92.35%)
PPV (95% CI)	66.67%	(49.10%-80.57%)
NPV (95% CI)	66.10%	(58.62%-72.86%)
PLR (95% CI)	2.61	(1.26-5.41)
NLR (95% CI)	0.67	(0.49-0.92)
Accuracy (95% CI)	66.27%	(55.05%-76.28%)

Abbreviations: PPV: Positive Predictive Value; NPV: Negative Predictive Value; PLR: Positive Likelihood Ratio; NLR: Negative Likelihood Ratio; CI: Confidence Interval

Diagnostic test with cut-off ≥ 7.440 ng/mL in the 2x2 table obtained sensitivity, specificity, PPV, NPV, PLR, NLR, and accuracy. The positive findings in this study are good specificity and predictive value. The specificity in this study was 82.98%, which means 82.98% of HBV DNA ≥ 20,000 IU/mL could be removed with a p22cr level < 7.440 ng/mL (Table 4). The specificity of this study is lower than Shimakawa *et al*., which got 92.9% specificity.[21] It could be due to false positive results. False positives in this study have been minimized by performing cautious sample handling during preparation and examination to prevent contamination. Increasing p22cr levels are not always in line with increased HBV DNA. This is in accordance with the research of Wong *et al*. who reported the detection of HBcrAg containing p22cr in post-therapy chronic hepatitis B patients with undetectable HBV DNA examination.[22] The PPV in this study was 66.67%, meaning if the p22cr level was ≥ 7.440 ng/mL, 66.67% of CHB patients will have HBV DNA ≥ 20,000 IU/mL. The NPV was 66.10%, meaning if the p22cr level was < 7.440 ng/mL, 66.10% of chronic hepatitis B patients will not have HBV DNA ≥ 20,000 IU/mL. PPV greater than NPV indicates that p22cr > 7.440 ng/mL can predict CHB patients with HBV DNA ≥ 20,000 IU/mL causing it necessary to initiate therapy. HBV DNA is important in determining the initiation therapy for CHB patients.[3,23] However, it has several limitations, such as expensive costs, limitations to large laboratories, and requires trained human resources. It can cause delays in therapy initiation causing complications such as hepatic cirrhosis or HCC to occur. The p22cr has been proven in previous studies, which reported that most of the Dane particles did not have HBV DNA but had a dominant p22cr and gave new hope in assessing cccDNA activity.[9] ELISA of p22cr is easier than PCR of HBV DNA so it can be an alternative examination of HBV DNA when it is not available in making decisions on therapy in patients with chronic hepatitis B.[24]

The sensitivity of p22cr was 44.44%, which means 44.44% of HBV DNA ≥ 20,000 IU/mL in CHB patients could be detected using p22cr level ≥ 7.440 ng/mL. The sensitivity of this study is lower than previous studies by Shimakawa *et al*. who found that the sensitivity of HBcrAg, which contains p22cr, in detecting HBV DNA ≥ 20,000 IU/mL was 88.9%.[21] The sensitivity in the previous study is higher because HBcrAg not only detects p22cr but can detect 2 other proteins from HBV, namely HBcAg and HBeAg.[8] The study also did not exclude HBeAg-positive subjects, so the sensitivity of HBcrAg could be higher. The low

sensitivity in the current study was due to the lack of patients with HBV DNA ≥ 20,000 IU/mL. This can be caused by several things, such as age, duration of hepatitis B, and HBeAg negative. Patients ≥ 60 years in this study were 21 (25.3%) patients and 15 (71.4%) had HBV DNA < 20,000 IU/mL. Jia *et al*. reported that older people with hepatitis B tended to have lower HBV DNA levels due to changes in liver function in the elderly.[25] Liver regeneration, cellular maintenance, and liver perfusion in the elderly will be disturbed impairing HBV replication in the liver. The duration of hepatitis B affected the HBV DNA level in relation to the hepatitis B phase.[15] Subjects in this study were mostly dominated on the border between the immune clearance and immune control phases, which were marked by a mean HBV DNA $2.9x10^3$ IU/mL, HBeAg negative, and laboratory parameters of liver disease progression that still showed normal values. The HBeAg negative, which was used as the study inclusion criteria also played a role. Chronic hepatitis B patients with HBeAg positive will have higher levels of HBV DNA.[25] The PLR in this study showed that the probability of HBV DNA ≥ 20,000 IU/mL in CHB patients when the p22cr level ≥ 7.440 ng/mL was 2.61. The NLR in this study showed that the probability of p22cr level < 7.440 ng/mL in CHB patients who have HBV DNA ≥ 20,000 IU/mL was 0.67. This study has poor PLR and NLR. A good diagnostic test has PLR >10 and NLR <0.1.[26] The accuracy in this study was 66.27%, which means if the p22cr examination was used in patients, 66.27% of the examination provide the correct conclusion in determining HBV DNA ≥ 20,000 IU/mL in CHB patients.

The limitation of this study is the use of p22cr, which is part of HBcrAg, so the use of HBcrAg as a marker might produce better diagnostic performance than p22cr. Elderly patients were included as study subjects. HBV replication slows down in the elderly, so this can affect the level of p22cr.[27]

CONCLUSIONS AND SUGGESTIONS

This study showed that the diagnostic performance of p22cr levels with a cut-off of 7.440 ng/mL had a sensitivity of 44.44% and specificity of 82.98% in detecting HBV DNA of 20,000 IU/mL in CHB patients. P22cr level has a good specificity so it can be an alternative examination of HBV DNA in making decisions on therapy in patients with CHB. Further research needs to be done using HBcrAg and excluding elderly patients.

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