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Conformity of Cleia and clia HBsAg Quantitative Assay with qPCR Gold Standard in Hepatitis B Patients

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***Abstract*---**Quantitative HBsAg assay is an alternative marker of potential viremia and monitoring response to antiviral treatment. Various methods have been developed to assess HBsAg quantification. This cross-sectional observational research aims to understand the performance of quantitative HBsAg assay with CLEIA (Chemiluminescence Enzyme Immunoassay) and CLIA (Chemiluminescence Immunoassay) methods and their compliance with qPCR. Serum samples were obtained from 69 Hepatitis B patients at the Outpatient Clinic. Quantitative HBsAg assay was performed with Sysmex HISCL-5000 and Mindray CL-900i, HBV DNA by using GeneXpert® HBV Viral Load. The results showed that the HBsAg value of the CLEIA method ranged from 9.77 to >2,500 IU/mL, while the CLIA method ranged from 19.97 to >50,000 IU/mL. Analysis of the CLEIA and CLIA methods showed agreement ((LoA: -5014.01 – 1815.33) with the relationship between the two methods showed a positive correlation ($p < 0.05$). There is a positive correlation between HBsAg results using CLEIA method and HBV DNA ($p < 0.05$) and also between the HBsAg CLIA method and HBV DNA ($p < 0.05$). In conclusion, both CLEIA and CLIA methods can be used to quantitatively assess HBsAg, however, they are not interchangeable for the evaluation of a patient due to differences in the detection ranges of the devices.

***Keywords*---**Quantitative HBsAg, CLEIA, CLIA, qPCR.

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Introduction

Hepatitis B virus (HBV) infection is the leading cause of the acute and chronic liver disease (e.g. liver cirrhosis and hepatocellular carcinoma) causing approximately 1.4 million deaths annually. It is estimated that currently around the world 2 billion people are infected with HBV and 248 million are chronic carriers of HBV Surface Antigen (HBsAg) (Fibriani *et al.*, 2014; Muljono, 2017; Nguyen *et al.*, 2020). The prevalence of Hepatitis B in Indonesia is 0.39% or 1,017,290 (Mulyatno *et al.*, 2009; Nelwan *et al.*, 2010). It was found that from biomedical examination of 10,391 serum samples, these samples had HBsAg positive prevalence of 8.4% (Utsumi *et al.*, 2013). Hepatitis B virus infection is spread mainly through percutaneous or mucosal exposure to infected blood and various body fluids, including saliva, vaginal fluids, and seminal fluid. Perinatal transmission is the main route of transmission of Hepatitis B virus infection. A person who is HBsAg positive has the potential to infect and transmit the virus to others (Tang *et al.*, 2019).

Hepatitis B virus is a type of double-stranded circular DNA virus surrounded by a hexagonal core region. This virus belongs to the *Hepadnaviridae* family (Humairah *et al.*, 2021; Nurtjahyani *et al.*, 2021). This virus has three specific antigens, namely surface, envelope, and core antigens. Hepatitis B surface antigen (HBsAg) is an antigen complex found on the surface of the hepatitis B virus. This viral infection has various serological markers other than HBsAg, namely Hepatitis B surface antibody (anti-HBs), Hepatitis B core antibody (anti-HBc), Hepatitis B envelope antigen (HBeAg), Hepatitis B envelope antibody (anti-HBe) and HBV DNA (Tang *et al.*, 2018). The incubation period for hepatitis B infection is 30-180 days, with an average of 70 days. HBsAg examination is the most frequently performed examination for the detection of hepatitis B infection. This is because HBsAg is the earliest indicator to appear prior to the presence of any clinical symptoms (Rinonce *et al.*, 2013; Utsumi *et al.*, 2010).

Testing and diagnosis of hepatitis B infection is the first step done in prevention and treatment services, and an important component of an effective response to the hepatitis epidemic. The most important diagnosis and screening for hepatitis B virus infection are HBsAg, because this antigen is the fastest detectable indicator in patients infected with HBV both acutely and chronically. Quantitative HBsAg assays are used to predict disease activity and monitor treatment response in chronic hepatitis B, especially in HBeAg-negative chronic HBV infection and in patients who are to be treated with interferon-alpha (IFNa) (Agarwal *et al.*, 2017). Measurement of HBsAg levels is standardized in IU/mL., and is now almost a mandatory measure due to the development of new antiviral treatments aiming HbSAg seroclearence, which is the functional cure of hepatitis B. HBsAg levels can aid immune tolerance differentiation and immune clearance in patients with Hepatitis B e antigen (HBeAg) positive, and can predict inactive disease and spontaneous HBsAg seroclearance in HBeAg negative patients (Cornberg *et al.*, 2017).

Measurement of serum HBV-DNA levels is very important for diagnosis, determination of stage of infection, treatment decision, and subsequent patient monitoring (Ie *et al.*, 2015; Muljono *et al.*, 2018). HBV replication rates represent

the single strongest predictive biomarker associated with disease progression and the long-term outcome of chronic HBV infection. Inhibition of viral replication by antiviral treatment has shown to achieve seroclearance of chronic HBV-induced necroinflammatory activity and progressive fibrotic liver processes in the majority of patients, in turn reducing the risk of liver cancer. The exact level of HBV DNA reduction that must be achieved for this benefit is still unknown, however, it is concluded that the lower the level, the better (Agarwal *et al.*, 2017). Molecular HBV DNA testing is expensive and unavailable at many health facilities. HBsAg quantification is an alternative marker for the indication of potential viremia and is used to monitor response to antiviral treatment (Rinonce *et al.*, 2013; Utsumi *et al.*, 2010).

Various methods have been developed for quantitative HBsAg measurement, including Chemiluminescence Micro Immunoassay (CMIA), Enzyme-Linked Fluorescent Assay (ELFA), Chemiluminescence Enzyme Immunoassay (CLEIA), and Chemiluminescence Immunoassay (CLIA). Each of these methods has advantages and disadvantages that need to be further investigated, considering the yet unknown universal cut-off value and uniformity of detection limits for quantitative HBsAg examination. This study aims to determine the performance of quantitative HBsAg assay using the Chemiluminescence Enzyme Immunoassay (CLEIA) and Chemiluminescence Immunoassay (CLIA) methods.

Method

This research has been approved by the Health Research Ethics Committee of Dr. Soetomo General Hospital, Surabaya. This research is an observational study using a cross-sectional design. The research was conducted at Dr. Soetomo General Hospital Surabaya from November 2019 to June 2020. The study population was patients suspected of having hepatitis B and patients who had a history of hepatitis B who came to the Gastroentero-hepatology Outpatient Clinic at Dr. Soetomo General Hospital. Inclusion criteria included male or female patients at the Gastroentero-hepatology Outpatient Clinic aged above 18 years. Patients with a history of hepatitis B immunization in the past 1 year were included in the exclusion criteria.

Serum samples were examined with Sysmex HISCL-5000 and Mindray CL-900i devices to obtain quantitative HBsAg levels. Measurement results are expressed in IU/mL. Sysmex HISCL-5000 was used to measure HBsAg, using the Chemiluminescence Enzyme Immunoassay (CLEIA) method. This device has an examination speed of approximately 17 minutes/test and 200 tests/hour. The measurement range of HBsAg concentration of this device is 0.03 – 2,500 IU/mL. The interpretation of the results of Sysmex HISCL-5000 is as follows:
- Non reactive if HBsAg concentration < 0.03 IU/mL.
- Reactive when HBsAg concentration ≥ 0.03 IU/mL.

The Mindray CL-900i measured HBsAg using the Chemiluminescent Immunoassay (CLIA) method with the sandwich immunoassay principle. The lowest detection limit of HBsAg concentration for this device is 0.05 IU/mL. The result of HBsAg concentration < 0.05 IU/mL means non-reactive, while concentration ≥ 0.05 IU/mL means reactive. The upper limit of the detection

range is 250 IU/mL. Samples with a yield > 250 IU/mL require dilution which can be done automatically or manually.

Discussion

This study obtained a total of 71 samples, 2 samples were excluded because subjects were under 18 years of age and the HBV DNA results were negative. All blood samples obtained were immediately processed in order to obtain serum samples. The serum was divided into 3 cuvettes of 0.5 - 1 mL each. All serum samples were then stored at -80^{o}C, before measuring the levels of HBsAg using each device and viral load on GeneXpert.

The characteristics of the samples are summarized in Table 1. The median age of the 69 samples was 33 years of age with the proportion of 56.5% of male subjects and 43.5% of female subjects. The HBsAg levels of all samples were measured using the CLEIA and CLIA methods. Amongst the 69 samples, the CLEIA method had the most samples with HBsAg levels ranging between 501-2,500 IU/mL, namely 30 samples. Meanwhile, the CLIA method had the most samples with HBsAg levels above 2,500 IU/mL, namely 43 samples. Of the 43 samples, 25 samples had HBsAg levels above 2,500 IU/mL using the CLEIA and CLIA methods. The results of the measurement of the HBsAg levels using the two methods are shown in Table 2.

Table 1
Characteristics of Samples

	n	**%**
Age (year) (n = 69)		
Mean ± standard deviation	36.58 ± 12.610	
Median (min – max)	33.0 (16 – 64)	
Sex		
Male	39	56.5
Female	30	43.5

Table 2
Measurement Levels of HBsAg using CLEIA and CLIA Methods

IU/mL	**HBsAg Method**			
	CLEIA		**CLIA**	
	n	**%**	**N**	**%**
< 50	5	7.2	2	2.9
50 – 250	4	5.8	7	10.1
251 – 500	5	7.2	1	1.4
501 – 2,500	30	43.5	16	23.2
> 2,500	25	36.2	43	62.3

The results of the normality test for HBsAg levels in this study were not normally distributed ($p < 0.05$). The suitability of the CLEIA method and CLIA method results were analyzed using Bland Altman. The mean difference

value was -1,599.34. The results of the analysis show that there is a match between the CLEIA and CLIA methods, where almost all of the measured HBsAg levels fall within the range (LoA: -5,104.01 -1,815.33). One outlier was found in the Bland Altman plot as shown in Figure 1.

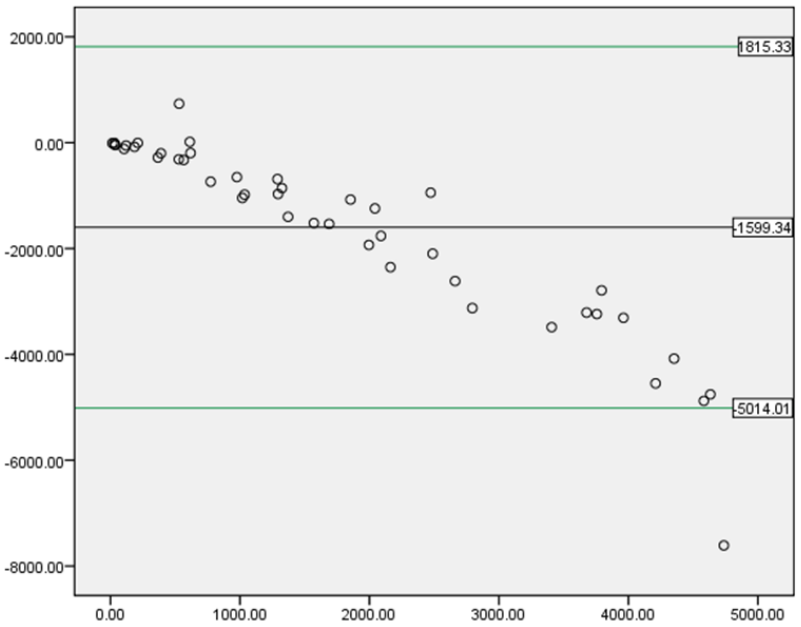

Figure 1. Results of the suitability test for HBsAg values using the CLEIA and CLIA methods with the Bland Altman plot

Kappa coefficient of 0.411 with a p-value of <0.001 ($p<0.05$) was obtained through the suitability test. According to the results, it can be said that there is conformity between the results of HBsAg obtained using the CLEIA method and the CLIA method, with a moderate level of conformity.

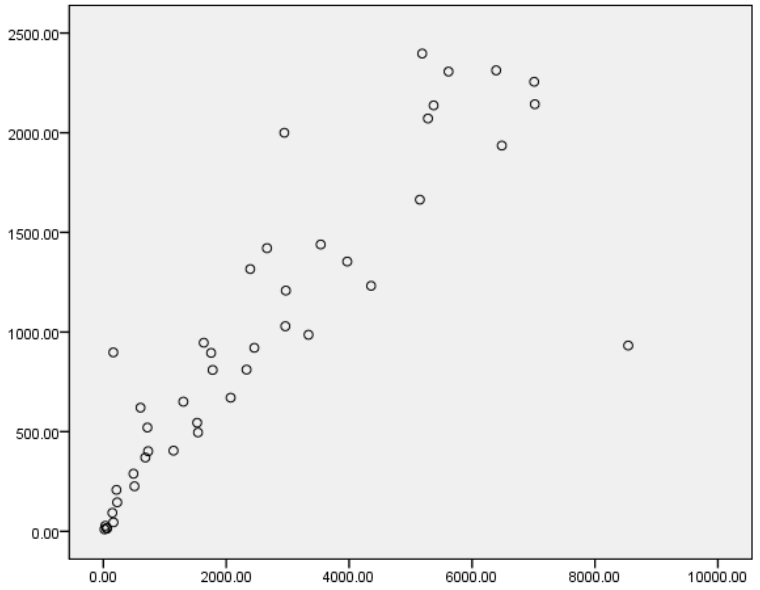

Figure 2. Spearman correlation test between HBsAg values obtained using CLEIA and CLIA methods

Correlation test was also performed on HBsAg levels obtained using CLEIA and CLIA methods, with the value of HBV DNA as measured by GeneXpert. The results of the Spearman correlation test showed that there was a significant correlation between HBsAg levels obtained using the CLEIA method and HBV DNA values ($p<0.05$), with a positive direction and strong relationship strength (0.6-0.8). These results were slightly different compared to the results of the Spearman correlation test between HBsAg levels obtained using CLIA method and HBV DNA values, which showed a significant correlation ($p<0.05$), positive relationship direction, and moderate relationship strength (0.4-0.6).

In patients with chronic hepatitis B infection, it is important to monitor the natural history, assess their response to therapy, and predict the possible risk of complications. Quantitative HBsAg assay plays a role in fulfilling these interests. The results of our study found that there was a match between the CLEIA and CLIA methods in measuring quantitative HBsAg. This is similar to a study by Deguchi *et al.* who found a match between CLEIA and CLIA, both of which also have a strong correlation (Deguchi *et al.*, 2018; Ie *et al.*, 2015; Muljono *et al.*, 2018).

There are differences in the results of HBsAg measurements using the CLEIA and CLIA methods. In the CLEIA method, the majority of the HBsAg levels obtained ranged between 251-2,500 IU/mL, while in the CLIA method, the majority of the HBsAg levels ranged above 2,500 IU/mL. A difference in the levels of HBsAg using the two methods. These differences are thought to be related to the sample pretreatment process and the use of antibodies against HBsAg, which contribute to the reduction of the effect of anti-HBs antibodies in the blood. We suspect that the difference in results in this study may be due to the different substrates of the two devices (Deguchi *et al.*, 2018; Purwono *et al.*, 2016; Yano *et al.*, 2015).

The lowest measurement results of HBsAg, which belonged to the <50 IU/mL group, were found to also be different between CLEIA and CLIA methods. Only five samples from the CLEIA method belonged to the <50 IU/mL group with the lowest value of 9.773 IU/mL. Meanwhile, there were only 2 samples from the CLIA method that belonged to the <50 IU/mL group, with the lowest value of 19.97 IU/mL. This difference can be related to the difference in the detection limits of the two devices, where the Sysmex HISCL-5000 has a lower detection limit than the Mindray CL-900i.

The relationship between quantitative HBsAg and HBV DNA results in HBV-infected patients is said to be complex and vary, depending on the treatment setting. In this study, 1 sample was excluded because the patient was found to be HBsAg-positive using CLEIA and CLIA methods, however, the result of HBV DNA was negative. This may occur in patients treated with nucleoside/nucleotide analogs. In addition, as HBsAg consists of the both virion and subviral particles, serum HBsAg levels are an expression of transcriptionally active intrahepatic covalently closed circular DNA (cccDNA), whereas serum HBV DNA levels indicate the amount of virion production (Deguchi *et al.*, 2018; Song and Kim, 2016).

The majority of new antiviral agents do not target the HBV polymerase, which makes serum HBV DNA insufficient to be used alone in monitoring the antiviral effect. Alternative markers are needed to reflect the effects of these agents on patients. Agents that cause a rapid decrease in serum HBsAg show promising HBsAg seroclearance levels. A decrease in serum HBsAg levels during PEG-IFN therapy can predict treatment-induced immune responses. In patients who do not achieve a sufficient reduction in serum HBsAg levels during the initial phase of PEG-IFN, further treatment is predicted to be unsuccessful and should be discontinued. International guidelines recommend that all chronic hepatitis B patients treated with PEG-IFN should receive regular monitoring of HBsAg levels at 3, 6, and 12 months while consuming PEG-IFN, and at 6 and 12 months post-treatment. The current recommendation for the use of HBsAg levels as a 'stopping rule' depends on the HBV genotype (Gunardi *et al.*, 2014; Mak *et al.*, 2020). Various studies have found a correlation between HBV DNA and quantitative HBsAg levels. The results of this study also found a correlation between HBV DNA and quantitative HBsAg levels as measured by the CLEIA and CLIA methods, with strong and moderate correlation strengths. The limitations of this study were the small number of samples, and in the CLEIA method, dilution was not performed. Further research by grouping samples at each phase of the HBV infection course with a larger number of samples, and performing dilution in all studied methods are needed to obtain better research results.

Conclusion

Quantitative HBsAg is an important marker of HBV, which reflects transcriptional activity with or without genome integration. Quantitative HBsAg levels are a promising diagnostic tool for monitoring the course of HBV infection before or during antiviral therapy, as HBsAg seroconversion and/or seroclearance are the ultimate goals of anti-HBV therapy. Quantitative HBsAg measurement can be used together with HBV DNA in determining the phase of hepatitis B disease,

predicting HBsAg seroclearance, knowing the need for treatment, evaluating and monitoring response to therapy, and understanding the risk of complications in the liver. Quantitative HBsAg measurements using CLEIA and CLIA methods are compatible, however, these methods cannot be used interchangeably or used together for the evaluation and monitoring of a patient due to the different detection ranges of each device.

Conflict of Interest

The authors declare that they have no conflict of interest in this study.

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