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DETERMINATION OF REACTIVE HBSAG CUT-OFF THAT NEED CONFIRMATORY TEST

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ABSTRACT

Hepatitis B surface antigen (HBsAg) is the earliest and most important serological marker for the diagnosis of HBV infection. The availability of new methods with a high sensitivity to detect HBsAg results in the increase of false reactive results so that a confirmatory test is needed,but this will increase the total test cost. A reactive cut-off value for a confirmatory test is needed to make the use of this test more efficient. This study was a cross-sectional. All the specimens with HBsAg >0.17 Cut-Off Index (COI) were confirmed with HBsAg confirmatory test. HBsAg test used a sandwich ELFA method while HBsAg confirmatory test used an antibody neutralization method. Analysis of the ROC curve obtained HBsAg cut-off value that need confirmatory test. Total samples were 80 with 51 (63.8%) confirmed reactive and 29 (36.2%) non-reactive. There was a statistically significant difference between HBsAg that confirmed reactive (median 2.76 COI) and non-reactive (median 0.32 COI) ($p<0.001$). ROC curve showed an AUC of 0.805 which meant a good diagnostic performance for HBsAg test based on a confirmatory test. The specificity of 89.66% and sensitivity 64.71% were obtained from the cut-off 1.08 COI and considered the best cut-off. Some possible causes of false reactive results were Hepatitis B vaccine, G-CSF therapy and limitation of the HBsAg methods. HBsAg cut-off with ELFA method that need HBsAg confirmatory test was <1.08 COI. The researchers suggests further studies with different sampling methods so a better data distribution can be obtained.

Key words: HBsAg, HBsAg confirmatory test, reactive cut-off

INTRODUCTION

Hepatitis B is an inflammation of hepatocytes caused by hepatitis B virus (HBV) infection. It is estimated that 240 million people around the world are having chronic hepatitis B infection and more than 686,000 people die every year due to hepatitis B complication.[1] Hepatitis B virus is a deoxyribonucleic acid (DNA) virus belonging to the hepadnaviridae family, size 42 nm that consists of 27 nm core nucleocapsids surrounded by a lipoprotein layer (envelope). HBV envelope contains surface antigen termed hepatitis B surface antigen (HBsAg) which is secreted into the bloodstream.[2] Serological markers for HBV infection consist of HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc IgM and IgG. The identification of serological markers allows identifying patients with HBV infection, to elucidate the natural course of Chronic Hepatitis B (CHB), to assess the clinical phases of infection and to monitor antiviral therapy. HBsAg is the earliest serological marker of HBV infection and plays an essential role in diagnosis. Accurate detection of HBsAg is crucial in early diagnosis and therapy.[3-5] After acute exposure to HBV, HBsAg appears in the serum within one to ten weeks. Persistence of this marker for more than six months implies chronic HBV infection.[6]

The availability of new methods with a high sensitivity to detect HBsAg results in increased false reactive results. HBsAg screening assays are generally supported by confirmatory tests, which are used to confirm repeatable reactive (positive) results. Typically the confirmatory test involves neutralization of the HBsAg in the sample by >50% using a human anti-HBs antibody.[7] In Western Europe and North America, the standard procedure for diagnosing HBV infection is to repeat and confirm test results in specimens with borderline levels of HBsAg and those with reactive results in initial testing.[4]

Confirmatory tests can be done using HBsAg Ultra Confirmation reagent to confirm a reactive HBsAg result from other standard methods. This confirmatory test is based on an antibody neutralization assay.[8] All available commercial HBsAg test kits emphasize the importance of confirmatory test for reactive HBsAg results.[9] Utilization of the neutralization test instead of HBV DNA test provides a cost saving for patients. The use of the neutralization test as a validation test when the HBsAg titer is less than or equal to a set limit will significantly reduce the cost of the test without the need for HBV DNA test.[10]

A study by Latuconsina determined the gray zone range that need a confirmatory analysis which was 0.13 – 0.17 COI. HBsAg value of more than 0.17 COI will be interpreted as reactive. All HBsAg values of more than 0.17 COI should be confirmed by a confirmatory test. However, if all the results have to be confirmed, it will greatly increase the overall cost so a cut-off for reactive HBsAg value that needs a confirmatory test is needed to make the use of this confirmatory test more efficient.[11]

Based on those backgrounds, this study was conducted to determine a reactive HBsAg cut-off using Enzyme Linked Fluorescent Assay (ELFA) that need a confirmatory test so it can be applied in the Clinical Pathology Laboratory of the Dr. Wahidin Sudirohusodo Hospital Makassar as well as other laboratories using the same methods.

METHODS

This study was cross-sectional which studied all specimens undergoing HBsAg initial test with ELFA methods in the Clinical Pathology Laboratory of the Dr. Wahidin Sudirohusodo Makasar from November 2015 to April 2017. The study samples included all specimens with an HBsAg initial test value >0.17 COI.

The 0.17 COI cut-off was used based on the cut-off applied in the Clinical Pathology Laboratory of the Dr. Wahidin Sudirohusodo Makasar. HBsAg detection used serum specimens, and sandwich ELFA methods (Vidas® HBsAg Ultra). All samples with HBsAg initial result >0.17 COI was subsequently confirmed by HBsAg confirmatory test. HBsAg confirmatory

Two measurements were done simultaneously as a confirmatory assay. The first measurement was done without addition of a confirmatory reagent containing anti-HBs while the second measurement was done with addition of a confirmatory reagent.

The reduction of the signal from the first measurement to the second measurement was calculated and expressed in percentage. HBsAg was considered as reactive if the signal reduction was more than or equal to 50%, while recognized as non-reactive if the reduction was less than 50%. Ethical clearance was obtained from the Commision of Medical Research Ethics, Faculty of Medicine, Hasanuddin University/Dr.Wahidin Sudirohusodo Hospital Makassar.

The HBsAg value difference between confirmed reactive and a non-reactive group was analyzed statistically using the Mann-Whitney test. An HBsAg cut-off that needed a confirmatory test was determined by Receiver Operating Characteristic (ROC) curve analysis. The results were presented in tables and graphs. Differences were considered statistically significant if the p-value was <0.05.

Table 1. Sample characteristics

Variables	n=80	HBsAg (COI) Median (min-max)
Age		
20 – 39	11 (13.75%)	0.93 (0.18 – 27.18)
40 – 59	38 (47.5%)	0.75 (0.18 – 26.11)
≥ 60	31 (38.75%)	0.79 (0.18 – 29.38)
Sex		
Male	50 (62.5%)	0.79 (0.18 – 29.38)
Female	30 (37.5%)	0.77 (0.18 – 26.11)

RESULTS AND DISCUSSION

Total specimens with HBsAg value >0.17 COI and confirmed with HBsAg confirmatory test was 80. The number of specimens from subjects aged 20-39 years was 11 (13.75%), 40-59 years was 38 (47.5%) and ≥ 60 years was 31 (38.75%). These results were consistent

Table 2. HBsAg value based on HBsAg confirmatory test results

	n = 80	HBsAg (COI) Median (min-max)	p*
HBsAg confirmatory test			
Reactive	51 (63.8 %)	2.76 (0.18 – 29.38)	<0.001
Non-reactive	29 (36.2 %)	0.32 (0.18 – 3.57)	

*Mann-Whitney test

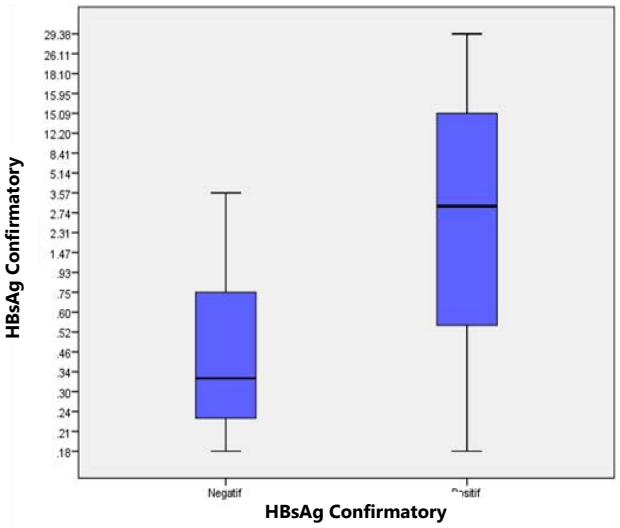

Figure 1. Box plot of HBsAg based on HBsAg confirmatory test results

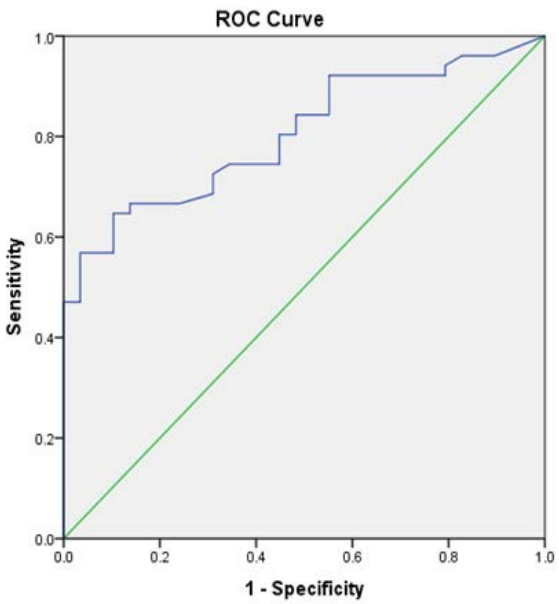

Figure 2. ROC curve of HBsAg value based on HBsAg confirmatory test results

with a report from Muljono stating that the highest prevalence of hepatitis B in Indonesia is in the age group 45-49 years.[12] Specimens from male subjects were 50 (62.5%) while from female subjects were 30 (37.5%). The highest number of samples was in the age group of 40-59 years and in the male group. The median of HBsAg value of different age and sex groups were not significantly different.

Table 2 showed the median of HBsAg value based on HBsAg confirmatory test results. The number of specimens confirmed reactive was 51 (63.8%) and non-reactive

Figure 2 was the ROC curve of HBsAg value based on HBsAg confirmatory test results. Area Under Curve (AUC) of the ROC curve was 0.805 which meant a good diagnostic performance for HBsAg test. Sensitivity and specificity with various cut-off values were determined using ROC curve analysis (Table 3).

The highest specificity, of 100%, was obtained from the cut-off of 4.095 COI with a sensitivity of 47.06%. It meant that all HBsAg results ≥ 4,095 COI would be confirmed as reactive and no false reactive results. However, the sensitivity at this cut-off was very low. The specificity of 89.66% with a sensitivity of 64.71% was obtained from the cut-off of 1.075 COI (≈ 1.08 COI). The researcher chose 1.08 COI as the HBsAg cut-off that needed a confirmatory test by considering the specificity and sensitivity obtained from ROC analysis. This cut-off determination was also considered the highest cost if we chose the cut-off with the highest specificity, which meant that more samples needed to be confirmed.

The HBV encodes the three proteins of the HBsAg, which form the viral envelope, small (SHBsAg), middle (MHBsAg) and large (LHBsAg). All three envelope proteins have a glycosylated form responsible for the secretion of viral particles. Serum HBsAg level could possibly reflect the amount and transcriptional activity of Covalently Closed Circular (CCC) DNA inside the hepatocytes. Detection of HBsAg is not only crucial for diagnosis but also for monitoring.[13]

Availability of various new methods with a high sensitivity to detect HBsAg, results in increased false reactive results. Our study also showed a high false reactive rate which is 36.2% of all reactive results. It leads to the importance of confirmatory test for reactive HBsAg results, and a reactive cut-off is required so that confirmatory test will be more efficient.[3,4,14] The researcher chose a reactive value of <1.08 COI as the reactive value that needed to be confirmed, with an expectation that it will give more accurate results with reasonable cost.

A study from Fletcher *et al.* stated that HBsAg values that need to be confirmed are weakly reactive values whereas highly reactive HBsAg values are not important for confirmatory testing.[3,14] No literature set HBsAg with ELFA methods cut-off for weakly reactive and highly reactive values so that each laboratory has to set its own HBsAg value cut-off that needs to be confirmed in order to reduce the possibility of false reactivity. Our results showed that a cut-off of 1.08 COI had the best specificity and sensitivity. It was consistent with the results of Fletcher *et al.* study which stated that confirmatory tests are only required for weakly reactive results.[3] Although there was no weak reactivity cut-off with the ELFA method, but the value of 1.08 COI could be classified as weakly reactive given that this value was in the low-value group of all the data obtained (0.18 - 29.38).

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Table 3. Specificity and sensitivity with various cut-off value

HBsAg cut-off	Specificity	Sensitivity
0.770	75.86	66.67
0.880	86.21	64.71
1.075	89.66	64.71
1.275	89.66	62.75
1.400	89.66	60.78
1.485	89.66	58.82
1.915	93.10	56.86
2.345	96.55	56.86
3.260	96.55	47.06
4.095	100.00	47.06

Rysgaard *et al.* concluded that one of the causes of false reactive HBsAg results was hepatitis B vaccination, although it generally only gave false reactive results up to 14 days post-vaccination. The study also concluded that weakly reactive HBsAg results often do not imply true

infection so confirmatory test is indispensable.[15] Fletcher *et al*. mentioned that false reactivity can be found in patients receiving Granulocyte-Colony Stimulating Factor (G-CSF).[3] Other causes described by Chen and Kaplan in their study, which stated that weakly reactive results were often false reactive due to the limitation of initial HBsAg test method which was the poor separation between signal and background noise. Chen and Kaplan's study was conducted on HBsAg test with the chemiluminescent method. The conclusion of the study also emphasized the need for each laboratory to evaluate the methods used.[16] Bigham and Ponnampalam study reported that influenza vaccine can possibly cause false reactivity in HBsAg test.[17] The causes of high false reactive rate from our data cannot be ascertained due to the limited information on vaccination and G-CSF therapy in our study subjects, but the false reactive results of a test was also a consequence of the higher test sensitivity.

The limitation of this study was the uneven distribution of HBsAg values. It was because sampling was performed on all specimens with HBsAg > 0.17 COI so that the HBsAg values obtained depended on the values in the population. More data obtained were at very low or very high HBsAg values, while fewer data in the 1-10 COI range.

CONCLUSION AND SUGGESTION

Based on the results of this study, the cut off value of HBsAg with ELFA methods that need HBsAg confirmatory test was <1.08 COI. HBsAg value of more than or equal to 1.08 COI is not necessary to be confirmed due to the high possibility to be confirmed reactive so that the test time and cost can be saved. The researcher suggests further studies with different sampling methods so a better data distribution can be obtained.

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