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Original article

Determination of initial HBsAg cutoff index threshold for reporting HBsAg reactivity

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Abstract

Background: Commercially available screening test for Hepatitis B surface antigen (HBsAg) are suggested as confirmatory tests in all reactive cases; however, reporting of results will take a long time, which increases the total test cost.

Objectives: To investigate the correlation between the cutoff index (COI) values of the initial HBsAg screening test and their corresponding confirmatory neutralization results to identify a COI value for distinguishing samples that truly need confirmatory neutralization tests.

Methods: This retrospective study analyzed 72,496 HBsAg screening test results using the Elecsys HBsAg II kit of patients between October 2019 and March 2022. Elecsys HBsAg II neutralization tests were performed on samples with COI values ranging from 0.9 to 30.0. The correlation between the COI value (0.9 - 30.0) and the percentage of neutralization confirmatory tests (HBsAg confirmed negative and positive groups) was plotted. The receiver operating characteristic (ROC) was also assessed.

Results: Of the 72,496 test results, 337 samples with COI ≤ 30.0 underwent confirmatory tests, yielding 313 positive samples. The ROC analysis revealed that the area under the curve was 0.9429 ($P < 0.0001$, 95% CI 0.8691 - 1) and the COI value of 3.5 had an excellent diagnostic value with the greatest positive likelihood ratio, providing 94.4% specificity (95% CI 74.2 - 99.7) and 88.3% sensitivity (95% CI 60.8 - 94.2). However, a high false-positive rate (11.7%) was found in samples with COI values of 0.9 - 4.0, whereas samples with values between > 4.00 and 13.00 had a 3.9% false-positive rate. On the contary, a COI value of 13.0 had 100.0% specificity.

Conclusion: In diagnostics, a COI value of ≥ 4.0 may be more practical in areas with limited resources for confirmatory tests. If the reasons for an increase in specificity outweigh the cost of confirmatory tests, a COI value of 13.0 would be more appropriate. Each diagnostic laboratory may choose the COI value that suits their settings for practical diagnostic application in areas with limited resources for confirmatory tests.

Keywords: Confirmatory neutralization test, cutoff index (COI), Hepatitis B surface antigen (HBsAg) screening, Hepatitis B virus (HBV) diagnosis.

In 2021, 1.5 million people were reported to have hepatitis B virus (HBV) infection.[1] HBV infection increases the risk of the host developing chronic liver diseases and hepatocellular carcinoma (HCC).[2, 3] Accurate and timely diagnosis of HBV infection allows infected individuals with infection to become aware of their HBV status and prevents further transmission to uninfected ones.

Individuals with HBV infection may be categorized into those with acute infection, which is self-limiting, and those that become chronic.[4] Chronic HBV infection is marked by a persistent HBV infection, defined by the presence of hepatitis B surface antigen (HBsAg) for > 6 months.[5, 6] Therefore, the detection of HBsAg serves as the initial screening test for the diagnosis of HBV infection. When performing the HBsAg screening test, the World Health Organization (WHO) guideline recommends using a one-assay strategy in countries where the prevalence of HBV-infection is > 0.4%. If prevalence is < 0.4%, then a two-assay strategy should be implemented.[7] The two-assay strategy recommends conducting a neutralization test as a confirmatory test when laboratory-based immunoassays are used as the first screening test. If

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rapid diagnostics (tests RDTs) are used in the first screening and the neutralization test is unavailable, then a second RDT assay can be used.[7]

Thailand is located in Southeast Asia, which has an average prevalence of HBV infection, as indicated by the seroprevalence of HBsAg in 4.5% of the Thai population who were born before the immunization program.[8, 9] Therefore, Thailand may technically implement the one-assay strategy for the diagnosis of HBV infection. However, commercially-available HBsAg screening tests, such as those from Roche Diagnostics, recommend a two-assay strategy by performing a confirmatory neutralization test in all cases with cutoff index (COI) values of > 0.9. [10, 11] This would be in discordance with the WHO guidelines as previously stated. This also causes a financial burden for patients and diagnostic laboratories and decreases the cost-effectiveness of the initial HBsAg screening test.

For these reasons, this study aimed to investigate the correlation between COI values of the initial HBsAg screening by the electrochemiluminescence immunoassay (ECLIA) and the results of the corresponding confirmatory neutralization tests. Thus, this retrospective study was conducted to determine the suitable COI values from the HBsAg initial test that required confirmatory HBsAg tests.

Materials and methods

Patient study population

This retrospective study recruited all patients from King Chulalongkorn Memorial Hospital (KCMH), Thai Red Cross Society, Thailand who underwent HBsAg screening between October 2019 and March 2022. In total, 72,496 patients were included. Blood samples from these individuals were subjected to HBsAg screening via ECLIA and samples with COI values ranging from 0.9 to 30.0 (borderline or weakly reactive results) were subjected to neutralization confirmation tests. The study protocol is shown in **Figure 1.** Patient data collection was performed in accordance with the Declaration of Helsinki and the study was approved by the Institutional Review Board Committee at the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no.1154/2022).

HBsAg screening test

Blood samples of patients were indicated for HBsAg initial screening using Elecsys HBsAg II kit (Roche Diagnostics GmbH, Mannheim, Germany) on the cobas e801 anlayzer following the manufacturer's instructions. Briefly, samples were mixed with antibody conjugates labeled with a biotin and ruthenium complex. The resulting antibody-antigen sandwich complexes were captured after washing with streptavidin-coated magnetic microparticles. When voltage was applied, a chemiluminescent signal was produced and measured using a photomultiplier. Results were determined automatically by the software by comparing the chemiluminescence signal obtained from the reaction product of the sample with the signal of the COI values previously obtained by calibration. COI values are interpreted as follows: nonreactive (< 0.9), borderline (0.9 and < 1.0), and reactive (1.0).

HBsAg confirmatory tests

HBsAg neutralization test was used as a confirmatory tests. The HBsAg neutralization assay was performed using the Elecsys HBsAg II Confirmatory kit (Roche Diagnostics GmbH, Mannheim, Germany) and cobas e801 analyzer. Samples with initial reactive results were used for the pretreatment step by mixing the samples with a confirmatory reagent (polyclonal anti-HBs antibodies). After that, the pretreated samples were further used for HBsAg detection. Samples were interpreted as positive when the HBsAg present in the samples were bound and formed HBsAg-polyclonal anti-HBs antibody complexes resulting in a COI confirmatory reaction value that decreased significantly to 60.0% of the COI control reaction. Samples were interpreted as negative if the COI confirmatory reaction value was $> 60.0\%$ when compared with the COI control reaction. The percentage value of each confirmation results was calculated as COI confirmatory reaction/COI control reaction] $\times$ 100.

Statistical analysis

Demographic data including gender, age, year of birth (before or after 1992, where an extended program on immunization (EPI) in Thailand was initiated) were collected. HBsAg COI values and the percentage of confirmation results from the confirmed HBV-negative and HBV-positive groups were plotted.

Categorical data including gender and birth year were shown as numbers and percentages. The Chi-square test was used to analyze the differences between the two groups. Continuous data including age, HBsAg COI values and percentages of

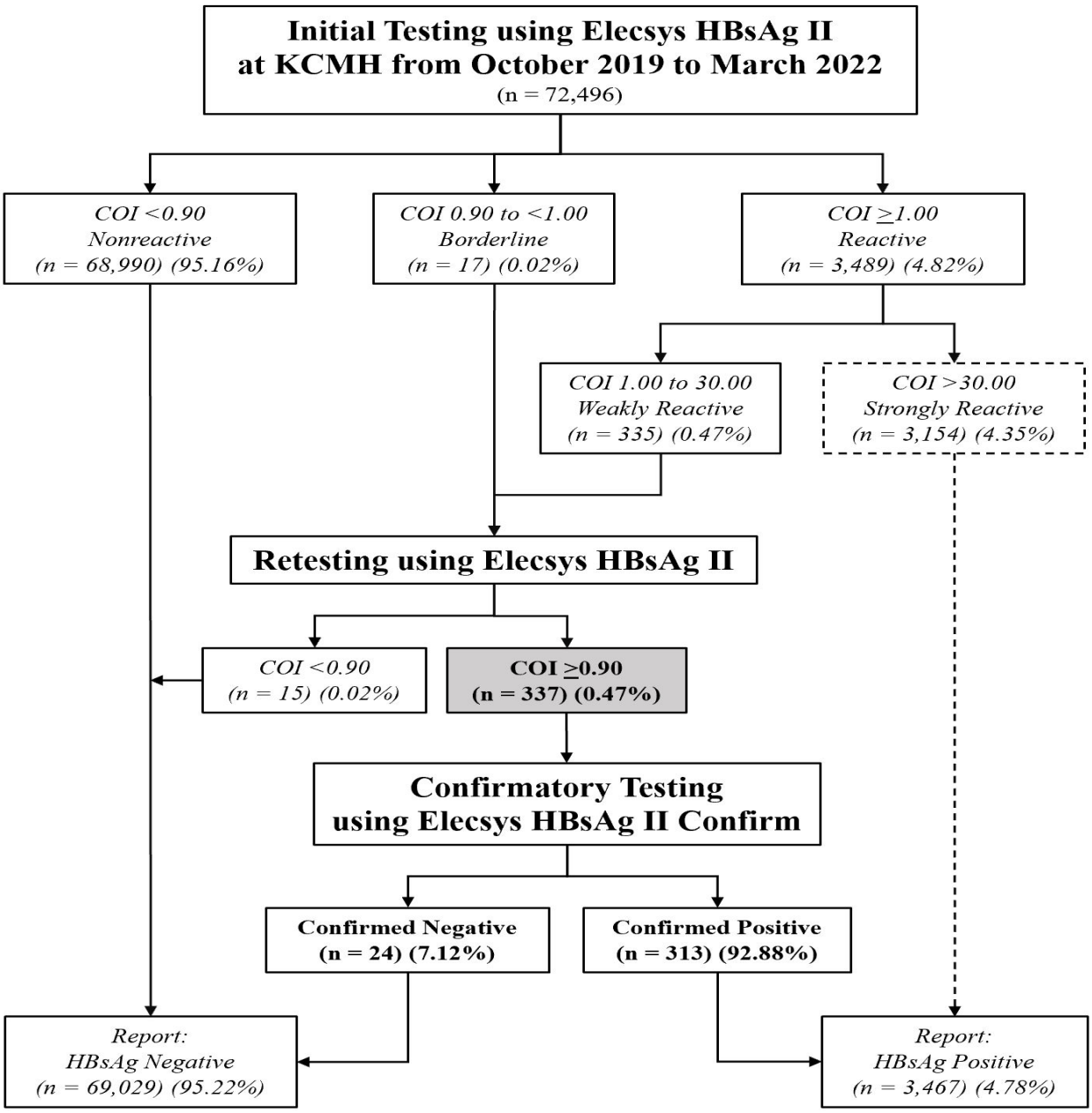

Figure 1. HBsAg testing algorithm and results from October 2019 to March 2022.

confirmation results were shown as numbers, mean values, standard deviations, medians, and ranges (min-max). The normality test was performed using the Kolmogorov-Smirnov test. Mann-Whitney U test was used to analyze the differences between the two groups. A receiver operating characteristic (ROC) curve was created to determine the appropriate HBsAg COI values to identify samples required for neutralization confirmatory tests. $P < 0.05$ was considered statistically significant. All statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA).

Table 1. Demographic and characteristics data of HBsAg confirmed negative and confirmed positive groups.

Variables	Total	Confirmed results by neutralization test		
		Negative	Positive	*P* - value
Total sample	**337**	24/337 (7.1%)	313/337 (92.9%)	
Gender				
Male	208 (61.7%)	14 (58.3%)	194 (62.0%)	0.7231[(a)]
Female	129 (38.3%)	10 (41.7%)	119 (38.0%)	
Total	**337 (100.0%)**	**24 (100.0%)**	**313 (100.0%)**	
Age (years)				
< 20	11 (3.3%)	2 (8.3%)	9 (2.8%)	
20 - 39	47 (14.0%)	12 (50.0%)	35 (11.2%)	
40 - 60	124 (36.8%)	5 (20.8%)	119 (38.0%)	
> 60	155 (46.0%)	5 (20.8%)	150 (47.9%)	
Total	**337 (100.0%)**	**24 (100.0%)**	**313 (100.0%)**	
Mean (SD)	57.0 (18.2)	43.4 (21.6)	58.0 (17.6)	
Median (Range)	59.0 (0 - 99)	36.5 (0 - 85)	60.0 (0 - 99)	0.0003*[(b)]
Birth				
Before EPI	308	16/308 (5.2%)	292/308 (94.8%)	< 0.0001*[(a)]
After EPI	29	8/29 (27.6%)	21/29 (72.4%)	
HBsAg COI Value				
Mean (SD)	7.2 (7.4)	2.9 (2.9)	7.5 (7.6)	
Median (Range)	4.6 (0.9 - 30.0)	1.6 (0.9 - 12.4)	4.7 (0.9 - 30.00)	< 0.0001*[(b)]
Percentage of confirmation				
Mean (SD)	19.8 (24.0)	96.0 (10.4)	13.9 (11.5)	
Median (Range)	11.3 (0.9 - 110.9)	98.1 (60.3 - 110.9)	10.2 (0.9 - 56.5)	< 0.0001*[(b)]

EPI, extended program of immunization; COI, cutoff index
* Statistically significant ($P < 0.05$), [a]*P* - value by Chi-square test,
[b]*P* - value by Mann-Whitney U Test

Results

HBsAg algorithm screening test in study population

A total of 72,496 HBsAg screening test results using the ECLIA of patients at KCMH between October 2019 and March 2022 were obtained. Test results were stratified based on COI values into nonreactive (COI < 0.9), borderline > 0.9 to < 1.0) and reactive (COI >1.0), with 68,990, 17 and 3,489 samples, respectively. We used our established in-house COI value to interpret the results as weakly reactive (1.0 < COI < 30.0) and strongly reactive (COI > 30.0). Cases considered strongly reactive were reported as HBsAg-positive without any additional tests. Cases with borderline and weakly reactive results were retested with a subsequent round of ECLIA (Elecsys HBsAg II). Samples with second COI values > 0.9 remained in the study, whereas those < 0.9 were excluded from further analysis. Retests of the 352 samples further excluded 15 test results that were initially in the borderline and weakly reactive group. Therefore, only 337 test results remained included in the study analysis. These 337 samples were subjected to neutralization confirmatory test. Results of the neutralization assay further classified the 337 test results into those as confirmed negative (n = 24) and positive (n = 313). **(Figure 1)**

Demographic data of total of 337 test results of confirmed negative and positive groups are shown in **Table 1**. The gender ratio in the confirmed negative group did not differ from that in the confirmed positive group ($P = 0.7231$). Most of the patients in the confirmed negative were 20 - 39 years old, whereas in the confirmed positive group, the majority of the patients were > 60 years old. Therefore, the median age was used to calculate the statistical difference in age between the confirmed positive and negative groups. Our analysis showed that both groups had significantly different median age ($P = 0.0003$). A higher percentage of confirmed negative cases were

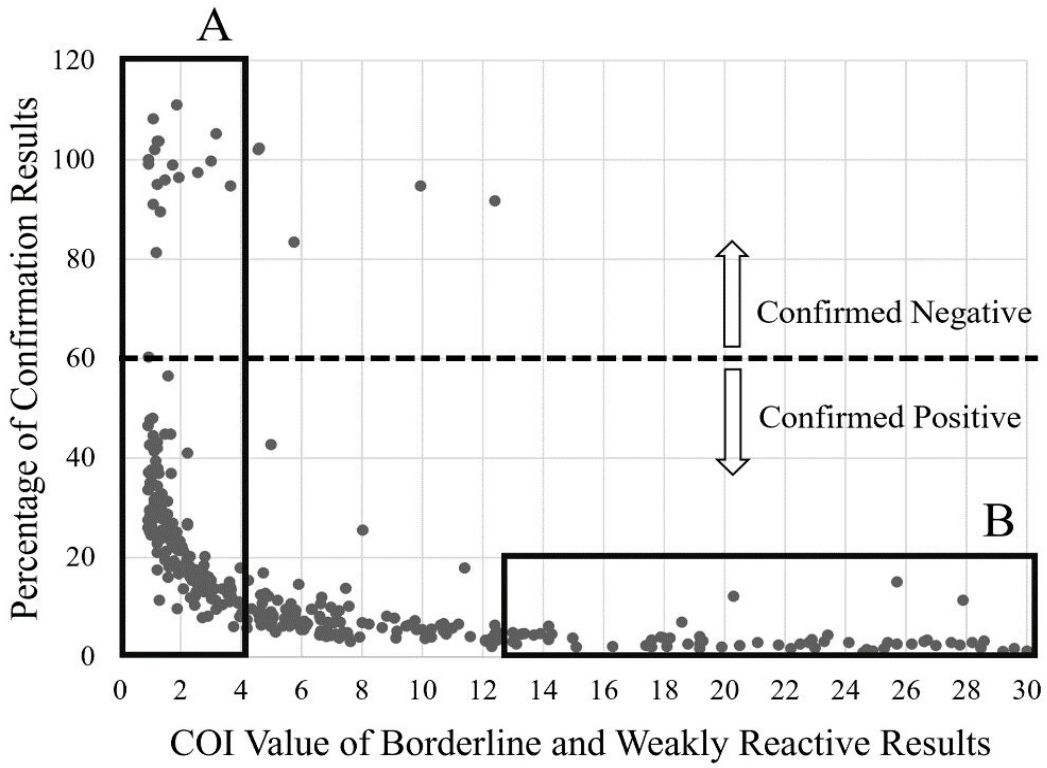

Figure 2. Correlation between the cutoff index (COI) value of borderline/weakly reactive results and the percentage of confirmation results.

found in cases established after the introduction of the EPI than in cases before its implementation. In addition, the HBsAg COI values and percentage value of confirmatory tests in the confirmed negative and positive groups were also significantly different ($P < 0.0001$ for both values).

Correlation between initial COI values and the percentage value of each neutralization test

We further correlated the initial HBsAg screening COI values of the 337 samples that were subjected to further confirmatory neutralization tests with their actual final results. The neutralization test results were utilized and its test result values (% neutralization) were correlated with the initial COI values of cases in the weakly reactive and strongly reactive groups. The majority of cases wherein results were confirmed negative from the neutralization tests were situated at the upper left quadrant area of the graph with the upper limit of confirmed negative results having initial COI values > 12 **(Figure 2, Table 2)**.

In addition, cases wherein results were confirmed positive from the neutralization tests that resembled a half hyperbola graph curve with cases that had initial COI values of < 4 had their neutralization test result values ranging from approximately 0.0 to 58.0% **(Figure 2).**

Cutoff COI values of 4 and 12 were then applied to stratify the test results into three groups (0.90 - 4.00, > 4.00 - 12.00, and > 12.00). A neutralization test result of > 60.0% was considered negative, whereas a test result of 60.0% or < 60.0% was considered positive. Samples with initial COI values 0.90 - 4.00, > 4.00 - 13.00, and > 13.00 had false-positive rates of 11.6% (19/164), 3.9% (5/113), and 0.0% (0/60), respectively **(Table 2).** This shows that as the initial COI value increase, the accuracy of the initial COI value reflects the actual results.

The Y axis depicts the percentage of the confirmation results from the confirmatory tests, wherein samples with a percentage > 60.0% were negative and samples with a percentage < 60.0% were

Table 2. Number of confirmed negative results, number of confirmed positive results used for receiver operating characteristic (ROC) analysis.

COI Value, range	Initial COI values with weakly reactive/borderline results (n)	Results confirmed negative (n)	Results confirmed positive (n)
< 1.00	12	3	9
1.00 - 1.99	84	12	72
2.00 - 2.99	43	1	42
3.00 - 3.99	24	3	21
4.00 - 4.99	25	2	23
5.00 - 5.99	19	1	18
6.00 - 6.99	19	0	19
7.00 - 7.99	14	0	14
8.00 - 8.99	5	0	5
9.00 - 9.99	8	1	7
10.00 - 10.99	9	0	9
11.00 - 11.99	4	0	4
12.00 - 12.99	11	1	10
13.00 - 13.99	8	0	8
14.00 - 14.99	3	0	3
15.00 - 15.99	2	0	2
16.00 - 16.99	1	0	1
17.00 - 30.00	46	0	46

COI, cutoff index

positive. COI values between 0.9 and 4.0 (A) yielded false-positive samples with a percentage > 60.0%. COI values ≥ 13.0 (B) were not false-positive samples.

Determination of HBsAg initial COI values

To determine the optimal COI value for the initial HBsAg screening tests, the sensitivity and specificity of each COI range were calculated **(Table 3)**. At one spectrum, the use of a low COI value showed high sensitivity but low specificity. In contrast, a high COI value (> 13) demonstrated low sensitivity but 100.0% specificity **(Table 3)**. An optimal diagnostic value requires a balanced between sensitivity and specificity. A COI value of 3.5 resulted in the highest likelihood ratio (LR) of 15.0, which gave 94.4% specificity (95% CI 74.2 - 99.7) and 88.3% sensitivity (95% CI 60.8 - 94.2) for the HBsAg screening test. Samples with initial COI values < 13 still showed false positivity **(Table 3).** Because of the disease burden of HBV infection and the purpose of using an initial HBsAg screening protocol without unnecessary confirmation tests to screen for HBV infection, a diagnostic test with a specificity of 100.0% was required. Therefore, COI values of > 13 may be used in this setting.

ROC analysis was performed using COI values between the confirmed negative results and positive results **(Figure 3)**. The area under the curve of the constructed ROC curve was 0.9429 ($P < 0.0001$, 95% CI 0.8691 - 1), indicating excellent diagnostic performance as an HBsAg initial test.

The ROC curve shows the correlation between the initial HBsAg COI values and the HBV-confirmed negative and the HBV-confirmed positive results with the area under the curve (AUC) of 0.9429 ($P < 0.0001$). The X-axis represents 1-specificity, the Y-axis represents sensitivity, and the area under the curve indicates the accuracy of the test.

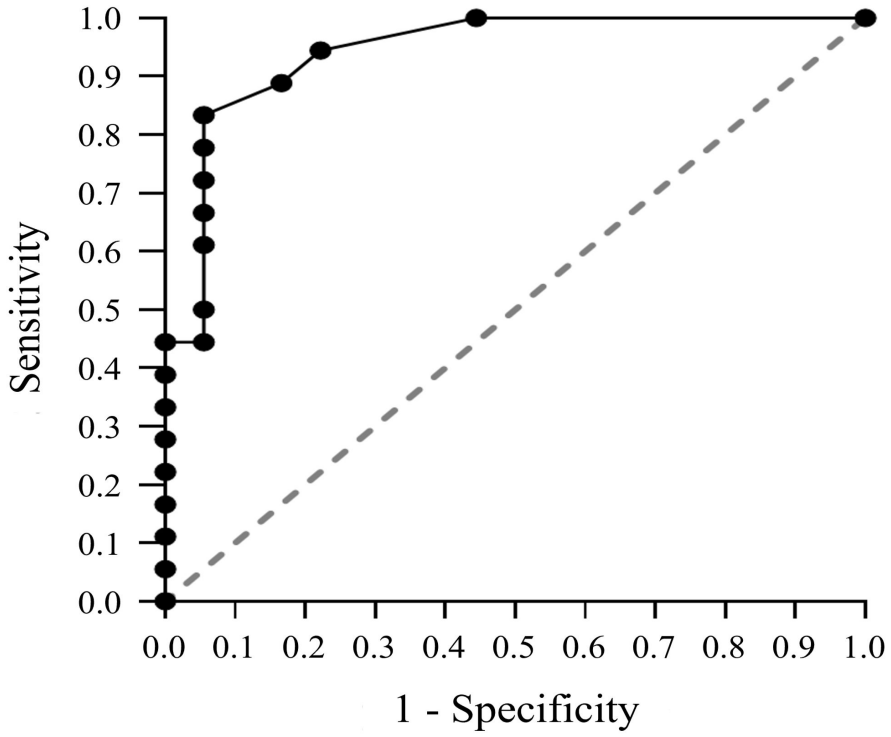

Figure 3. Receiver operating characteristic curve predicts the suitable cutoff index value for HBV infection identification.

Table 3. Sensitivity and specificity with various cutoff index (COI) value.

COI values	Sensitivity		Specificity		LR+
	Percentage	95% CI	Percentage	95% CI	
0.50	100.0	82.4 - 100.0	55.6	33.7 - 75.4	2.3
1.50	94.4	74.2 - 99.7	77.8	54.8 - 91.0	4.3
2.50	88.9	67.2 - 98.0	83.3	60.8 - 94.2	5.3
3.50	83.3	60.8 - 94.2	94.4	74.2 - 99.7	15.0
4.50	77.8	54.8 - 91.0	94.4	74.3 - 99.7	14.0
6.00	72.2	49.1 - 87.5	94.4	74.2 - 99.7	13.0
7.50	66.7	43.8 - 83.7	94.4	74.2 - 99.7	12.0
8.50	61.1	38.6 - 79.7	94.4	74.2 - 99.7	11.0
9.50	50.0	29.0 - 71.0	94.4	74.2 - 99.7	9.0
11.00	44.4	24.6 - 66.3	94.4	74.2 - 99.7	8.0
13.00	44.4	24.6 - 66.3	100.0	82.4 - 100.0	
16.00	38.9	20.3 - 61.4	100.0	82.4 - 100.0	
18.50	33.3	16.3 - 56.3	100.0	82.4 - 100.0	
20.00	27.8	12.5 - 50.9	100.0	82.4 - 100.0	
22.00	22.2	9.0 - 45.2	100.0	82.4 - 100.0	
32.50	16.7	5.8 - 39.2	100.0	82.4 - 100.0	

CI, confidence interval; LR+, positive likelihood ratio.

Discussion

The pooled prevalence estimation of hepatitis B infection in Thailand was approximately 5.0%; however, it was higher in men who have sex with men (8.1%) and people living with HIV (8.1%).[12, 13] Prompt and cost-effective tests help identify individuals with HBV infection who need treatment and surveillance to prevent horizontal transmission.

In a given algorithm for diagnostics, an initial screening test is usually performed with a high sensitivity assay to reduce false negativity and confirmatory tests with high specificity to correctly exclude disease-free individuals.[14] For the diagnosis of HBV infection, a confirmatory neutralization test is recommended by manufacturers to validate all initial HBsAg results that are considered reactive. The need to perform confirmatory tests is a financial and technical burden on both patients and diagnostic laboratories. In addition, regions that are considered to have an HBV infection prevalence of > 0.4% can be considered to have used only one assay when testing for HBV infection.[7] The determination of a suitable cutoff value may minimize the number of patients who need of subsequent confirmatory tests, increasing the total cost-effectiveness of the screening tests, and reducing the cost and turnaround time for diagnosing HBV infection.[15]

Therefore, in this study, we aimed to determine practical COI values of the initial HBsAg screening test that will distinguish between patients who will benefit from a confirmatory test and those wherein confirmatory tests will not change any patient management based on initial HBsAg screening results. COI values of initial HBsAg screening from 72,496 patients were categorized into nonreactive, borderline, weakly reactive, and strongly reactive results. Those with borderline and weakly reactive results further underwent repeated tests and those with a consistent COI value of > 0.9 were further included in the study analysis. A total of 337 tests results were correlated with their confirmatory neutralization tests into confirmed negative or positive groups. The confirmatory neutralization test indeed gave both confirmed negative and positive results to cases with initial COI values of < 13. Once initial COI values were above 13, confirmed neutralization test all gave the same results as the confirmed negative group. This suggests that in cases with initial COI values from the HBsAg screening test, 8.7% of cases (23/266) will be diagnosed as not reactive for HBV infection and will not require long-term treatment. This study showed that increasing the initial COI value to 13.0 (originally from 0.9 based on the manufacturer's recommendations) resulted in 100.0% specificity of the initial test and the absence of false positivity. Because of the chronicity of HBV infection, it increases the risk of HCC, even a low percentage of patients that can be excluded from long-term treatment will be beneficially financially, socially and mentally.

However, the cutoff values in this study may not apply to other areas with varying disease prevalence. The false-positive rate of the HBsAg initial test in this study was 7.1%; however, studies from Korea and China have reported false-positive rates as high as 41.0% - 43.0% among samples with low COI values or weak reactive results.[11, 16] The suitable cutoff values should be evaluated in each area, particularly when employing reagents from various manufacturers. Healthy people who had recently received vaccination may be HBsAg positive.[17] In this study, individuals who were included in the neutralization confirmatory tests who were born after the introduction of the EPI, which includes HBV vaccination, demonstrated a higher false-positive rate than those born before the EPI (27.6% *vs.* 5.1%, respectively). Screening tests for HBV infection that used a cutoff value higher than that in the manufacturer's recommended values with 100.0% specificity would aid in reducing samples requiring confirmatory tests.[16]

Conclusion

This study demonstrated that 93.0% of cases with equivocal HBsAg results were positive by confirmatory tests, having an overall false-positive rate of 7.0%. However, the false-positive rate was not distributed evenly among the range of initial COI values. The majority of cases with false-positive results had initial COI values ranging from 0.9 to 4.0. For practical application in diagnostics, in areas with low resources for performing neutralization confirmatory tests, a COI value of ≥ 4.0 may be more practical as only 3.9% of cases will be false positive, meaning that 96.1% of cases with initial COI values between > 4.0 and < 13.0 will not require a subsequent neutralization confirmatory tests. However, if the reasons to increase the specificity outweighs the cost to perform neutralization confirmatory tests, then COI value of 13.0 would be more appropriate. In addition,

having any COI value would also mean that not all tests will be subjected to the neutralization confirmatory tests. Nonetheless, it would also be possible to remain with the one-strategy approach to screen for HBsAg and accept the false-positive rate. However, HBV is a long-term infection, requires long-term treatment and is transmitted sexually. This affacts how HBV-diagnosed patients would maintain their lifestyle. Misdiagnosis will affect the quality of life of the patients.

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Conflict of interest statement

All authors has completed an ICMJE disclosure form. None of the authors declare any potential or actual relationship, activity, or interest related to the content of this article.

Data sharing statement

All data generated or analyzed during the present study are included in this published article and the citations herein. Further details, opinions, and interpretations are available from the corresponding author on reasonable request.

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