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SHORT COMMUNICATION

Enhanced Diagnostic Accuracy in Hepatitis B: Human vs. Sheep-Derived Antibodies in HBsAg Confirmatory Testing

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SUMMARY

Background: **HBsAg confirmatory testing is crucial for accurately diagnosing HBV infection and avoiding false positives. We compared the performance of human-derived and sheep-derived anti-HBs reagents in the Elecsys HBsAg confirmatory test (Roche Diagnostics, Germany).**
Methods: **Samples with a HBsAg COI of 0.9 or more underwent confirmatory testing with both reagents. Results showed a high overall agreement rate of 98% between human and sheep reagents.**
Results: **The average confirmation percentage with sheep anti-HBs was significantly lower than with human anti-HBs (6.9% vs. 20.0%, p = 0.002). Additionally, some strongly positive HBsAg samples could not be neutralized.**
Conclusions: **This suggests that the polyclonal antibodies in these reagents have different specificities and affinities for HBsAg epitopes. The findings emphasize the importance of reagent specificity and affinity to ensure accurate HBV diagnosis. Additional HBV markers and clinical context must be considered to confirm the diagnosis.**
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INTRODUCTION

Hepatitis B surface antigen (HBsAg) is the earliest serological marker to appear in the event of Hepatitis B virus (HBV) infection and is crucial for the diagnosis of HBV infection [1]. With the introduction of more sensitive immunological assays, the false positive rate for HBsAg testing has increased. To simplify the testing process while improving accuracy, the World Health Organization (WHO) recommends that laboratories adopt standardized HBsAg testing strategies, one-assay serological testing strategy or two-assay serological testing strategy [2].

The one-assay serological testing strategy is a fast and economical method that uses only one type of serological test to report HBsAg results, but it may report false positives. The two-assay serological testing strategy involves sequentially using two different types of serolo-

gical tests, where a positive result from the first serological test is confirmed with another serological test of similar sensitivity or through a neutralization test before reporting.

In the two-assay serological testing strategy, the neutralization test involves neutralizing the HBsAg in the patient sample with a reagent containing antibodies against it. The confirmation of HBsAg positivity is determined by the percentage decrease in the HBsAg test result value of the neutralized sample compared to the original sample's test result value.

Since January 2024, HBsAg neutralization tests for weakly positive HBsAg samples have been included in Korean national health insurance coverage. Contrary to insurance reimbursement criteria (weakly positive HBsAg samples), most manufacturers recommend conducting an HBsAg neutralization test on all samples that initially test positive for HBsAg. There are reports suggesting that confirmatory tests are particularly useful for identifying HBsAg false positives in weakly positive samples [3-6]. However, little is known about confirmation in strongly positive HBsAg samples. Recently, the reagent composition for the Elecsys HBsAg confirmatory test (Roche Diagnostics, Mannheim, Germany) has been updated from human-derived anti-HBs antibodies to sheep-derived anti-HBs antibodies.

In this study, we compared and evaluated the human anti-HBs confirmatory test with the sheep anti-HBs confirmatory test. This study is the first, to our knowledge, to evaluate the performance of the new Elecsys HBsAg confirmatory test by comparing human-derived and sheep-derived reagents. Unlike previous studies on HBsAg neutralization assays, we included very high-concentration HBsAg positive samples in our analysis.

MATERIALS AND METHODS

From March to May 2023, HBsAg positive samples were used to conduct and compare confirmatory tests with human anti-HBs and sheep anti-HBs antibodies. A total of 50 samples were tested.

Initially, HBsAg was measured using the Elecsys HBsAg II (Roche Diagnostics) on the Roche cobas e801 analyzer. Elecsys HBsAg II is an electrochemiluminescence immunoassay that qualitatively detects HBsAg. A cutoff index (COI) result of less than 0.9 is considered HBsAg negative, a COI between 0.9 and less than 1.0 is considered borderline, and a COI of 1.0 or above is considered positive. The manufacturer recommends that all initially reactive or borderline samples should be retested in duplicate. If the sample COI is 0.9 or more in the duplicate retest, the sample should be investigated using a confirmatory test.

For the Elecsys HBsAg confirmatory test, repeatedly reactive HBsAg samples are treated in parallel with confirmatory reagent and control reagent (270 µL sample + 30 µL confirmatory or control reagent, respectively) and then incubated. The anti-HBs antibodies in the confirmatory reagent neutralize HBsAg in the sample, whereas the control reagent does not neutralize the sample. The previous Elecsys HBsAg confirmatory test used human anti-HBs antibody > 200,000 IU/L in human serum as the confirmatory reagent and human serum with anti-HBs antibody < 3 IU/L as the control reagent [7]. The new Elecsys HBsAg confirmatory test uses sheep anti-HBs antibody ≥ 500,000 IU/L in sheep serum as the confirmatory reagent and sheep serum negative for anti-HBs as the control reagent [8]. Samples treated with each reagent undergo an HBsAg assay. This leads to a reduction in the COI in the sample treated with the confirmatory reagent compared to the COI obtained from the sample treated with the control reagent. The confirmation (%) was calculated as follows:

$$Confirmation\ (\%) = \frac{HBsAg\ (COI)_{treated\ with\ confirmatory\ reagent}}{HBsAg\ (COI)_{treated\ with\ control}}\ x\ 100$$

If confirmation (%) is ≤ 60, the result is determined as positive for HBsAg. If confirmation (%) is > 60, the result is determined as negative for HBsAg.

RESULTS

Out of a total of 50 samples tested, the HBsAg COI of these samples ranged from 1.1 to 3,271. The total agreement rate between confirmatory test results performed with human anti-HBs and sheep anti-HBs was 98.0% (49/50) (Figure 1). Among these samples, six were weakly positive for HBsAg, with COI less than 10.0. Three (3/50, 6.0%) were found to be both negative with human and sheep derived confirmatory test (Table 1). There was one discrepant case (Table 1, case 4) showing a negative result in the human anti-HBs confirmatory assay (confirmation 78.8%) and a positive result in the sheep anti-HBs confirmatory assay (confirmation 40.3%). Case 1 was suspected of HBsAg false positivity because the HBsAg level was very low at 1.1 COI, and all other HBV serologic markers also tested negative. Cases 2 and 3 were considered true positive cases with high HBsAg results (339 COI and 2,122 COI for cases 2 and 3, respectively) and other positive HBV serologic markers. Case 4 is considered truly HBsAg positive, given the high HBsAg results (2,165 COI), positive anti-HBe antibody, and positive anti-HBc antibody results. Interestingly, it was found that the level of neutralization during the confirmation reaction varied between the two reagents. Even for the same patient, the neutralization levels differed when using human anti-HBs and sheep anti-HBs (Figure 2). The average sheep anti-HBs confirmation (6.9%) was significantly lower than average human anti-HBs confirmation (20.0%) ($p = 0.002$) according to a paired *t*-test, which means sheep anti-HBs neutralized human HBsAg better than human anti-HBs.

Table 1. Confirmatory test results and HBV serologic markers for cases showing negative HBsAg confirmatory test results.

Case No.	Sample HBsAg (COI)	Confirmatory test with human anti-HBs				Confirmatory test with sheep anti-HBs				Anti-HBs	Anti-HBe	HBeAg	Anti-HBc total
		Confirmation reaction (COI)	Control reaction (COI)	Confirmation (%)	Interpre-tation	Confirmation reaction (COI)	Control reaction (COI)	Confirmation (%)	Interpre-tation				
1	1.1	1.01	1.00	101.2	neg	1.04	1.00	104.0	neg	neg			neg
2	339	380	398	95.5	neg	374	373	100.3	neg	neg	neg	pos	pos
3	2,122	1,952	2,298	84.9	neg	1,414	2,213	63.9	neg	pos (19.8)			
4 [a]	2,165	1,784	2,264	78.8	neg	905	2,245	40.3	pos	neg	pos	neg	pos

[a] **Case 4 showed negative confirmation on human anti-HBs reagent and positive on sheep anti-HBs reagent.**
Anti-HBc - hepatitis B core antibody, Anti-HBe - hepatitis B e antibody, anti-HBs - hepatitis B surface antibody, COI - cutoff index, HBeAg - hepatitis B e antigen, HBsAg - hepatitis B surface antigen, neg - negative, No. - number, pos - positive.

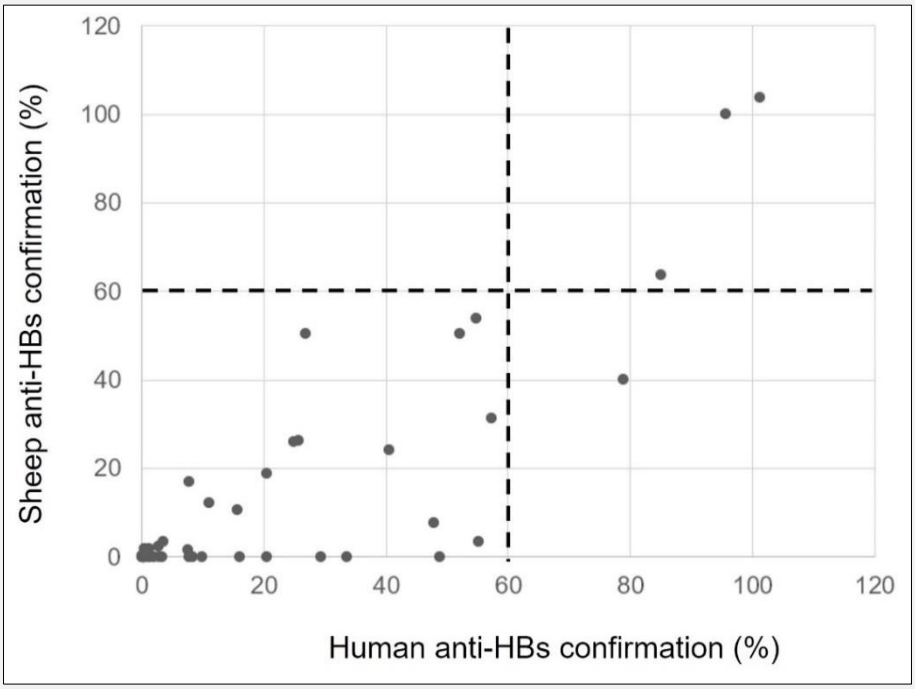

Figure 1. Four quadrant plot showing the distribution of Elecsys HBsAg confirmatory test between human anti-HBs and sheep anti-HBs.

DISCUSSION

Non-neutralization in weakly positive samples is considered a false positive result of the HBsAg test [9,10]. Reports of non-neutralization by confirmatory test in HBsAg strongly positive samples are scarce. In cases 2 and 3, where strongly positive HBsAg samples were not neutralized, the following situations, which indicate reduced neutralization capability of anti-HBs, should be considered: 1) immune escape mutants during chronic HBV infection [9], 2) superinfection with a new HBV strain [10], and 3) HBV reactivation during occult HBV infection in immunosuppressive conditions [11]. The non-neutralization observed in strongly positive HBsAg samples suggests that even high HBsAg levels do not guarantee successful neutralization. In such cases, additional HBV markers and clinical context must be considered to confirm the diagnosis.

The varying levels of neutralization that the polyclonal antibodies present in the two confirmatory test reagents have different specificities in binding to specific epitopes on HBsAg and exhibit varying affinities towards these epitopes. While HBsAg is known to have high heterogeneity, some conserved regions do exist [12]. The HBsAg is composed of the large, middle, and small hepatitis B surface proteins. The region of 99 to 169 amino acids is referred to as the main hydrophilic region within the small hepatitis B surface proteins. Within this region, amino acids 124 - 147 are specifically referred to as the 'a' determinant, which is a dominant neutralizing epitope [13]. Antibodies utilized in commercial immunoassays typically identify and attach to this specific area [14]. When the source of the reagent changes, especially to one derived from animal components, animal-derived antibodies might have different specificities and sensitivities towards human antigens due to differences in the epitope recognition between species [15]. Naturally produced human antibodies are likely to demonstrate high specificity and affinity for target molecules in human samples. Conversely, sheep antibodies, typically produced in the laboratory through immunization with antigens derived from different species, may possess a different specificity and affinity profile, sometimes recognizing a broader range of antigens.

One limitation of our study is the relatively small sample size, which may not capture the full spectrum of variability in HBsAg levels and antibody responses. We did not perform dilution for samples with highly positive HBsAg, and a hook effect is possible. Future research should include larger cohorts to validate these findings and explore the molecular basis for the observ-

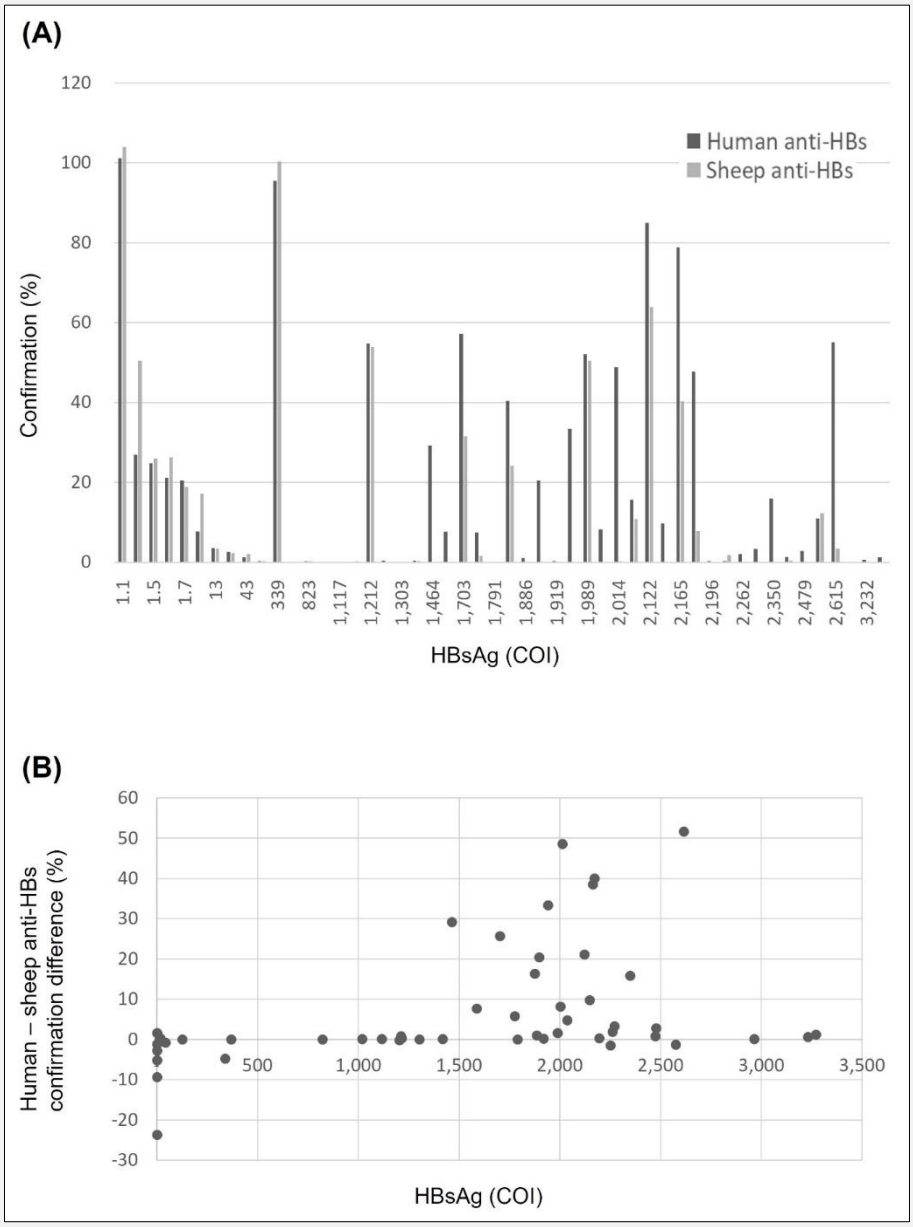

Figure 2. Differences between Elecsys human anti-HBs and sheep anti-HBs confirmatory tests.

(A) Comparison of human anti-HBs and sheep anti-HBs confirmation (%) according to HBsAg COI. (B) Human anti-HBs confirmation (%) - sheep anti-HBs confirmation (%) according to HBsAg COI.
anti-HBs - hepatitis B surface antibody, COI - cutoff index, HBsAg - hepatitis B surface antigen.

ed differences in epitope recognition and neutralization efficacy.

In conclusion, our study demonstrates that both human and sheep anti-HBs reagents show high concordance in HBsAg confirmatory testing, although significant differences in neutralization levels were observed. This indicates that sheep-derived antibodies have a higher neutralization capability compared to human-derived antibodies. Importantly, our findings reveal that some strongly positive HBsAg samples could not be neutralized, which underscores the necessity of considering reagent specificity and affinity in confirmatory testing.

Additional HBV markers and clinical context must be considered to confirm the diagnosis.

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This research received no external funding.

Ethics Statement:
This study was approved by the Ewha Womans University Mokdong Hospital Institutional Review Board and the requirement for informed consent was waived (approval number: EUMC 2023-03-037).

Declaration of Interest:
The authors have no potential conflicts of interest to disclose.

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