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Comparative Evaluation of ELISA and Immunochromatographic Test for HBsAg Detection: A Study of Sensitivity and Specificity

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Abstract

This study evaluates and compares the effectiveness of two diagnostic methods for detecting Hepatitis B surface antigen (HBsAg) are EnzymeLinked Immunosorbent Assay (ELISA) and the iminochromatographic test. ELISA, a widely utilized laboratory technique, offers high sensitivity and specificity but necessitates specialized equipment and technical expertise. In contrast, the iminochromatographic test is a rapid method that provides quick, user-friendly results, though its accuracy can vary. Evaluation, sensitivity, specificity of two methods were assesedusing a patient cohort. Our findings indicate that while ELISA remains the gold standard for HBsAg detection, the iminochromatographic test is a reliable alternative in resource-limited settings, albeit with slightly lower sensitivity. This comparative analysis underscores the importance of selecting diagnostic tools based on context, balancing accuracy with accessibility.

Key Words : HBsAg, ELISA, Immunochromatograph.

Introduction

Hepatitis B virus (HBV) causes serum hepatitis, impacting a significant number of people worldwide. Among those infected, 1-5% become chronic carriers. Carriers of Hepatitis B secrete the Hepatitis B surface antigen (HBsAg) into their blood and other bodily fluids, thereby increasing the risk of transmission to seronegative individuals.Thehepadnavirus family, to which HBV belongs, causes chronic hepatitis, and HBsAg is a crucial seromarker for early detection, appearing weeks before symptoms or abnormal ALT levels manifest. The incubation period of the virus ranges from 40 days to 6 months. HBV exhibits antigenic variability, with the primary forms being adw, adr, ayw, and ayr. The common "a" antigen in these particles is associated with unique determinants "d" or "y" and "w" or "r." Hepatitis causes inflammation of the liver, an essential organ responsible for defending against infections, filtering blood, and processing nutrients. Causes include pollutants, alcohol, drugs, and medical conditions. There are five main hepatitis viruses (A, B, C, D, and E), each with significant health impacts due to disease burden, mortality, and potential for outbreaks.[1]

Hepatitis B is a viral infection that specifically targets the liver, potentially causing both acute and chronic illnesses. The virus spreads through contact with infected blood and bodily fluids, with higher risks during childbirth, sexual activities, and unsafe injection practices. According to the WHO, hepatitis B affects 296 million people worldwide, with 1.5 million new chronic cases emerging each year. In 2019, hepatitis B-related complications, such as cirrhosis and liver cancer, caused 8.2 Lakh deaths. Despite the availability of safe and effective vaccines that offer 98-100% protection, hepatitis B continues to pose a significant public health challenge. The WHO Western Pacific and African regions experience the highest burden of the disease, with millions chronically infected. Preventive measures include vaccination, safe sexual practices, and proper handling of blood. Diagnosis involves blood tests, liver ultrasounds, and biopsies. Treatments for chronic hepatitis B include antiviral drugs and interferon injections, with liver transplantation as an option for severe cases. Additionally, coinfection with HIV complicates hepatitis B management, as 2.7 million people are affected by both viruses.[2-4]

Enzyme-Linked Immunosorbent Assay (ELISA), is an immunoassay technique used for detecting diseases.ELISA operates on the principle that specific antibodies can attach to a target antigen, allowing for the detection and quantification of these antigens. To enhance the assay's sensitivity and precision, plates are coated with high-affinity antibodies. This method provides a valuable measurement of antigen-antibody concentrations.[5,6,7 8,9]

Types of ELISA

In Direct ELISA, the enzyme is directly linked to the antibody that attaches to the antigen on a surface. In Indirect ELISA, a primary antibody binds to the target antigen, and a secondary antibody with an enzyme conjugate detects the bound primary antibody. Sandwich ELISA involves capturing the target antigen between two antibodies, forming a complex. Competitive ELISA measures antigen concentration by detecting the competition between the target antigen and a labeled antigen for binding to a specific antibody[10-13]. Types of ELISA are depicted in fig 1.

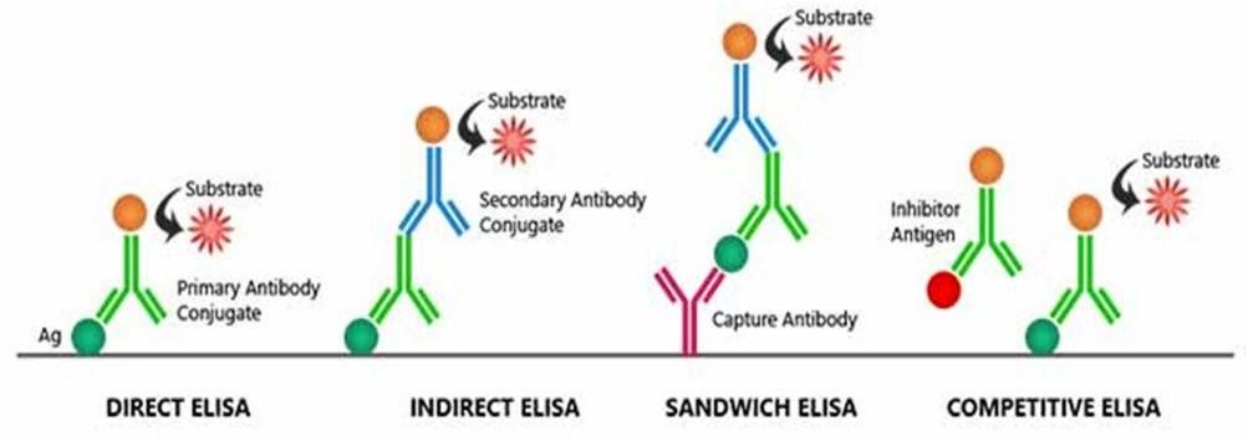

Fig 1. Types of ELISA

Principle of HEPALISA

Based on the "Direct Sandwich" theory, HEPALISA is a solid-phase enzyme-linked immunosorbent assay (ELISA) as depicted in Fig. 2. Microwells are coated with monoclonal antibodies that are highly reactive to HBsAg. When samples are added to the wells, an enzyme conjugate composed of polyclonal antibodies linked to HRPO is also introduced. In the wells, HBsAg from the serum sample is "trapped" or "sandwiched" between the monoclonal antibody and the HRPO-conjugated polyclonal antibody, forming a sandwich complex. Unbound conjugate is washed away with a buffer. The amount of bound peroxidase is directly proportional to the concentration of HBsAg in the sample. A chromogen and substrate buffer are added, producing a blue color. The intensity of this blue color indicates the amount of HBsAg present in the sample. When the stop solution is added,

the enzyme-substrate reaction stops, turning the color to yellow. This yellow color is then measured spectrophotometrically at 450 nm.

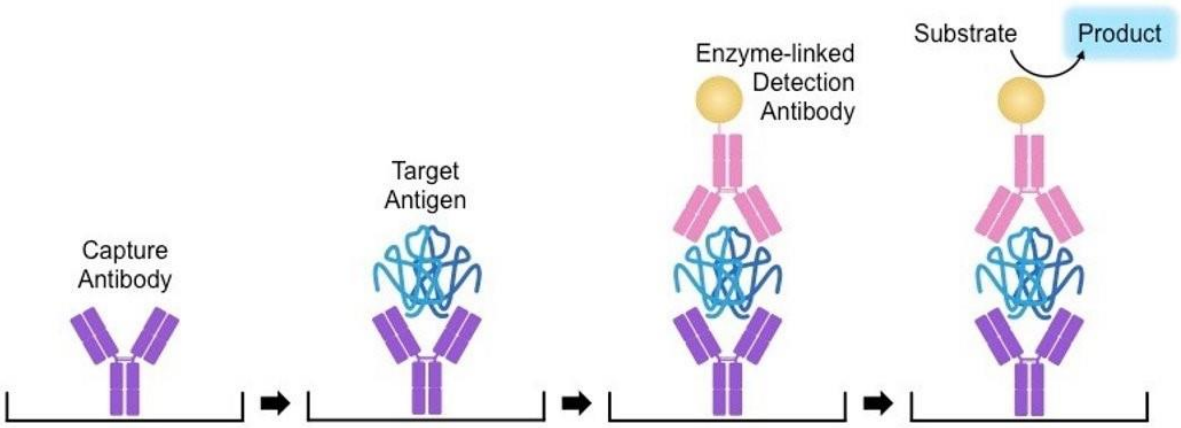

Fig 2. Sandwich method of HEPALISA

Immunchromatography

Immunochromatography, or lateral flow immunoassay (LFIA), is a rapid, on site diagnostic method for detecting HBsAg. It involves applying a small sample to a test strip with specific antibodies, producing a visible color change within 15-30 minutes if HBsAg is present shown in fig 3. While this method is simple, fast, and ideal for resource-limited settings, its sensitivity and specificity are generally lower than ELISA, necessitating confirmation of positive results with more precise laboratory. The Bsic components of Immunochromatography is represented in table 1.

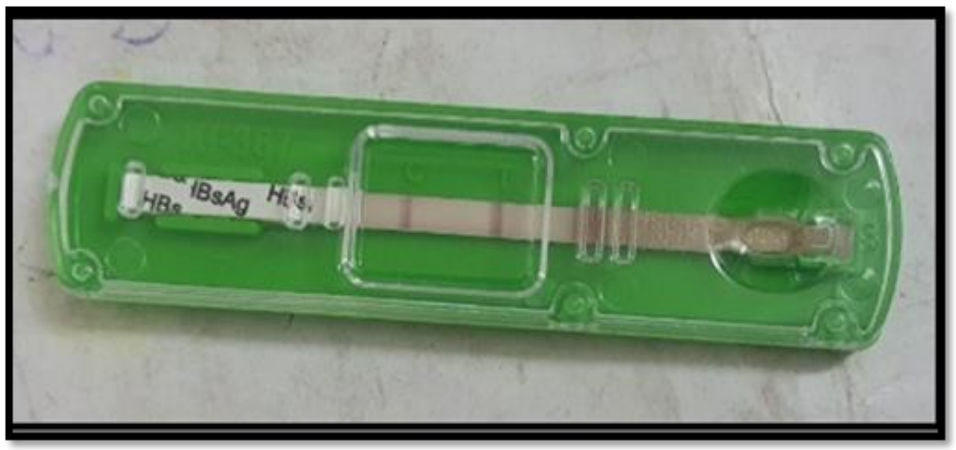

Fig 3. Immunochromatographic strip

Table 1. Components of a standard Immunochromatographic strip

Sample Application Pad:	
Composition	Composed of cellulose or glass fibers.
Function	Starts the assay upon application of the sample.
Purpose	Migrates the sample to other components smoothly, steadily,

	and homogeneously.
Pretreatment	Include seperation of sample components, removal of interferences, adjust pHetc.
Conjugate Pad:	
Purpose	Dispenses labeled antibodies, typically nano colloid gold particles.
Function	Releases the labeled conjugate when the liquid sample begins to flow.
Stability	The labeled conjugate must remain stable for the entire duration of the lateral flow strip.
Impact	Proper distribution, drying, and release of the conjugate significantly affect the assay's results and sensitivity.
Materials	Made from polyester, cellulose, and glass fiber, among others.
Nitrocellulose Membrane:	
Sensitivity	Crucial to the sensitivity of the immunochromatographic assay (ICA).
Markings:	Has markings for test and control lines.
Binding	Should facilitate strong binding to capture probes, such as antibodies.
Non-Specific Adsorption	Should exhibit minimal non-specific adsorption in the test and control line sections.
Dispensing	Correct dispensing, drying, and blocking of bioreagents are essential for improved sensitivity.
Adsorbent Pad:	
Function	As a sink at the end of the strip.
Flow Regulation	Regulates the flow rate of liquid over the membrane and prevents sample backflow.

Principle of ICT

The HBsAg Test cassette features a membrane strip pre-coated with a mouse monoclonal anti-HBs capture antibody in the test band region. When the sample is applied, the mouse monoclonal anti-HBs-colloid gold conjugate and the serum sample travel chromatographically along the membrane. This movement forms an antibody-antigen-antibody gold particle complex in the test region (T), resulting in a visible line, as shown in Fig. 4. The surface of the HBsAg Test cassette is marked with "T" for the Test Line and "C" for the Control Line. Prior to applying any samples, the Test and Control lines are not visible in the result window. If the test procedure is performed correctly and the control line reagents are functioning, the control line should always appear.

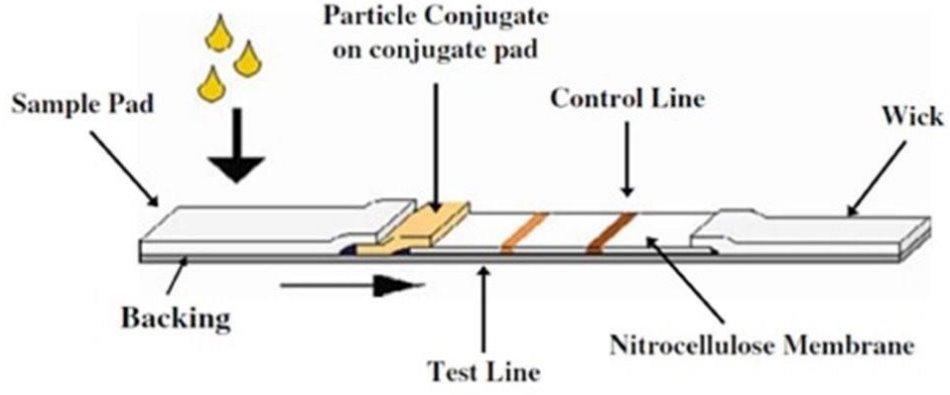

Fig 4. Cross-section view of Immunochromatographic strip

Materials and Methods

Materials

Purpose

Hepalisa detects HBsAg in human serum or plasma in vitro qualitatively. Before transfusion, it is primarily used as a screening test.

The Hepatitis B Virus Serological Markers [14]

HBsAg:

HBsAg is detected during the incubation period, typically 6-8 weeks before symptoms appear. The symptoms can appear within 2 weeks of exposure and usually disappears within 3-4 months. The HBsAg test is a reliable and universal indicator of HBV infection. In chronic carriers and cases, it persists for more than six months.

Anti-HBs:

HBsAg clearance appears several weeks to months later, causing a 'window' period. It is a good indicator of HBV immunity and recovery. Quantitation assists in pre-vaccination screening and post-vaccination monitoring.

HBeAg:

It appears within a week of HBsAg, disappears before HBsAg clearance, and returns within 3-6 weeks of HBsAg clearance. It is indicative of a highly infectious state, except in precore mutants. A persistent infection over a period of 10 weeks may suggest chronic infection or hepatitis.

Anti-HBe:

It Appears before HBsAg clearance, indicating decreasing infectivity and a favorable prognosis.

IgM Anti-HBc:

During the acute stage, it is found at high titers during the serological "window," then declines as the patient recovers. A marker of recent infection, distinguishing acute from chronic hepatitis.

Anti-HBc Total:

It appears 4-10 weeks after HBsAg and persists for many years. It is an indicator of HBV exposure during the window period and a prominent indicator of HBV exposure.

HBV-DNA:

A low-level PCR detection facilitates the diagnosis of acute or chronic infections, as well as monitoring the response to interferon treatment. In addition to the above antigens and viral DNA polymerase, it is useful as a diagnostic marker for HBV infection.

ELISA Kit

The Hepalisa in-vitro diagnostic kit detects the surface antigen (HBsAg) of Hepatitis B in human serum and plasma. It is used primarily as a screening test for blood collected prior to transfusion. The components of the kit are detailed in Table 2.

Table 2. ELISA Kit & Its Components

Particular	Description	Quantity
Microwells	Anti-HBsAg (monoclonal) coated microwells packed in a desiccant pouch.	5 Vials
Enzyme Conjugate Concentration (50%)	Anti-HBsAg polyclonal IgG linked to horseradish peroxidase with protein stabilizers.	1 Vial (0.25 ml)

Conjugate Diluent	Buffer containing stabilizers.	1 Bottle (9 ml)
Wash Buffer Concentration (25%)	Concentrated Phosphate buffer with surfactant.	1 Bottle (35 ml)
TMB Substrate	To be diluted with TMB diluent before use.	1 Bottle (10 ml)
TMB Diluent	Buffer solution containing H_2O_2 with preservative	1 Bottle (10 ml)
-ve Control	An anti-HBsAg, anti-HCV, anti-HIV serum that is ready to use.	1 Vial (2.0 ml)
+ve Control	A ready-to-use, inactivated and diluted human serum that reacts with HBsAg and is non-reactive with HIV and HCV.	1 Vial (2.0 ml)
Stop Solution	Ready to use, 1N H_2SO_4	1 Vial (15 ml)
Plate Sealers	Microwells are covered with adhesive sheets during incubation.	4 Nos.

Storage and Stability
Components should be stored at 2-8ºC. Do not use any components beyond their expiry date.

Glassware and Instruments
Micropipettes&microtips, ELISA reader, distilled water, graduated cylinders, absorbent tissues, timer, ELISA washer, Incubator (37^0C), disposable gloves and disinfectant solution.

Method

Specimen collection & handling:
The test should only be performed on human serum or plasma samples. To prevent hemolysis, promptly extract the serum from the clot during sample processing. Fresh serum or plasma samples are preferred. The specimens must be free from microbiological contamination and can be stored frozen at -20°C or lower, or between 2-8°C for up to one week. Repeated freezing and thawing and usage of heat-inactivated samples must be avaoided to yield inaccurate results. Samples that are icteric, hemolyzed, or hyperlipemic can also lead to erroneous findings.Sodium azide should not be used as a preservative as it can inactivate horseradish peroxidase.

Specimen Processing (Frozen Sample HEPALISA):
Thaw the frozen sample in a vertical position to let particles settle at the bottom. Centrifuge the sample for 15 minutes, after thawing.

Preparation of reagents:
The incubator is preheated to 37°C. In order to prevent condensation, let the foil pack containing the microwell strips reach room temperature (20-25°C). Place the strips in the strip holder after breaking off the required number of strips. In addition to the negative and positive controls for each run, include at least one negative and one positive control for each additional strip. Unused wells should be stored at 2-8°C with desiccant enclosed in aluminum sacks secured with clamps and rods.Microwells remain stable for 30 days when stored properly.

Preparation of Working Wash Buffer:
Check for salt crystals in the wash buffer concentration. If present, wash and resolubilize the solution by heating to 37°C until the crystals dissolve completely.Prepare 25 milliliters of working wash buffer for every strip used (1 milliliter of concentrated wash buffer with 24 milliliters of water). Thoroughly mix before use.Combine 480 ml of deionized or distilled water with 20 ml of the 25X wash buffer concentration. When stored between 2 and 8°C, Working Wash Buffer remains stable for two months.

Preparation of Working Conjugate and Working Substrate Solution:

Dilute conjugate concentrate 1:50 in conjugate diluent. Prepare a fresh dilution for each assay and mix thoroughly before use.Mix TMB substrate and TMB Diluent in a 1:1 ratio to prepare the working substrate solution are represented in Table 3.

Table 3. Preparation of working conjugate and working substrate solution

Reagents	**Well-1**	**Well-2**	**Well-3**	**Well-4**	**Well-5**
Samples	Sample –1 (100µl)	Sample –2 (100µl)	Sample –3 (100µl)	Positive control (100µl)	Negative control (100µl)
Enzyme conjugate	50µl	50µl	50µl	50µl	50µl
TMB substrate solution	100µl	100µl	100µl	100µl	100µl
Stop solution	100µl	100µl	100µl	100µl	100µl

Procedure:

ELISA PROCEDURE FLOW CHART[15,16,17]

At initially add 100 µl of controls and samples to the coated microwells.

↓

Prepare working conjugates

↓

Add 50 µl of working conjugate to the vials

↓

Cover the vials and incubate at 37°C for 60 min (fig 5-7)

↓

Wash the vials in plates for 6 cycles

↓

Prepare chromogenic substrate

↓

Add 100 µl of substrate to all the vials

↓

Incubate in dark for 30 min at room temperature (fig 8)

↓

Add stop solution (fig. 9)

Read results at 450 nm and 630 nm(fig 10)

Fig 5. Microtiter plate

Fig 6. Wells with positive and Negative control

Fig 7. wells with conjugate enzyme

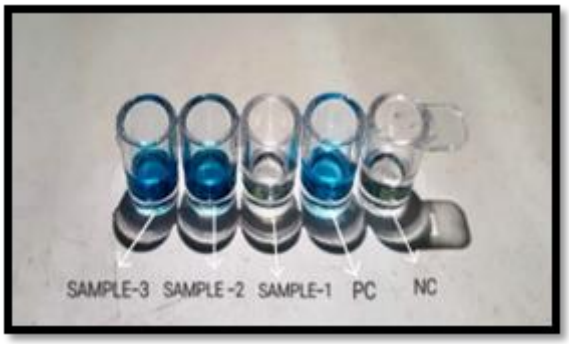

Fig 8. Wells after incubation

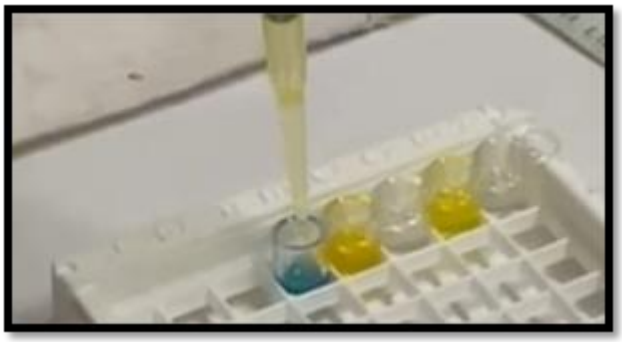

Fig 9. Adding stop solution (H_2SO_4)

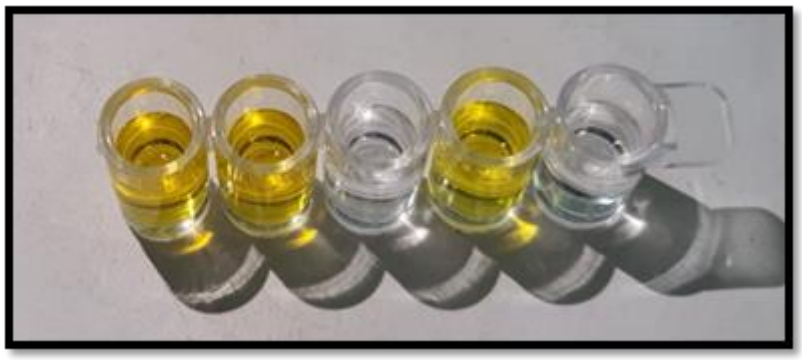

Fig 10. Wells with end of the reaction.

Limitation of the test:

The test is intended for the qualitative detection of HBsAg only in serum or plasma samples. Other bodily fluids should not be tested.The presence or absence of Hepatitis B Surface Antigen in the specimen is the sole outcome of this screening test. A final diagnosis should consider the patient's clinical history, symptoms, and serological information. Reactive samples must be validated by a confirmatory test before reporting results.False positive results may occur in the presence of RF antibodies, autoimmune diseases, liver or renal issues, and prenatal samples.

Immunochromatogram:

Specimen collection and storage:

Plasma specimens should be collected in tubes containing anticoagulants, such as heparin, EDTA, or sodium citrate. Centrifuge at 5000 rpm for 15 minutes.For serum specimens, draw whole blood into a tube without anticoagulants. Coagulate for 30 minutes, then centrifuge.Store serum between 2-8°C. If not tested immediately, freeze at -20°C or below if kept for more than three days.

Test Procedure:

Place the pouch at room temperature.Check the expiry date and packaging integrity. Do not use if expired or damaged.Open the cassette and check the desiccant. Use only if the color remains unchanged.Place the cassette on a clean, level surface and use immediately.Add 60 µl (2 drops) of serum or plasma to the sample window and allow it to soak in.Interpret results within 20 minutes. Do not interpret after 25 minutes.

3.3.8. Limitations of the test:

The HBsAg Rapid Test Kit is designed to screen for HBsAg in serum or plasma. PCR or ELISA tests should be used to confirm reactive samples. The assay is validated exclusively for individual serum and plasma samples, not for pooled samples or other types of fluids. Strict adherence to the test procedure is crucial for obtaining accurate results. A non-reactive result does not rule out Hepatitis B virus exposure or infection. Patients with autoimmune liver diseases may exhibit falsely reactive results due to non-specific sample binding.

Results & Discussion:

Interpretation of result of ELISA:

Absorbance (OD) values below the cutoff are considered non-reactive or negative for HBsAg. Absorbance values at or above the cutoff are considered reactive for HBsAg. If the absorbance value of a specimen falls within 10% of the cutoff, the specimen should be retested in triplicate. According to HEPALISA's criteria, specimens with absorbance values at or above the cutoff are initially reactive and require retesting. If both duplicate retest samples have absorbance values below the cutoff, the specimen is considered non-reactive. A specimen is deemed reactive if any duplicate retest sample has an absorbance value at or above the cutoff, or if both duplicate retest samples meet this criterion. Confirmatory assays or further testing are advised.[18-23]

Performance Characteristics of ELISA:

Analytical Sensitivity: The kit's sensitivity, using the WHO standard, is 0.250 IU/ml. Sensitivity to 0.1 ng/ml is observed for all 11 subtypes, including mutant strains.
Subtype Specificity: Hepalisa reacts with various HBsAg subtypes, including ayw1, ayw2, ayw3, ayw4, ayr, adw2, adw4, adrq+adyw, adr(q neg), and ayw3.Sensitivity and Specificity Assessment: WHO evaluated Hepalisa's performance in terms of sensitivity and specificity, obtaining 100% for both.Precision: Within-run and between-run precisions show coefficients of variation (%) within 10%.

Interpretation of the results of Immunochromatogram:

Negative: Only the control line appears, indicating HBsAg concentration is below the detection limit.Positive: Both control and test lines appear, indicating a positive result.Invalid: Absence of the control line renders the test invalid, requiring repetition following the exact procedure.

Performance characteristics of Immunochromatography:

Sensitivity & Specificity:In-house Evaluation: Sensitivity and specificity were compared to a reference test equipment, yielding less than 100%.
External Evaluation by NIB India: Sensitivity and specificity were both 100%.
Comparison with ELISA:The immunochromatographic test demonstrated a sensitivity of 96.8% and a specificity of 99.7%. Despite differences in measuring absorbance, the immunochromatogram's positive/negative result correlates with ELISA values.The comparative values of ELISA and Imunochromatographyare represented in table 4 and Fig 11.

Table 4. Absorbance values of ELISA and Immunochromatography

Samples	Absorbance values	
	ELISA (AU)	**Assumed Immunochromatography (AU)**
Sample - 1	1.289	1.35
Sample - 2	1.302	1.35
Sample - 3	0.009	0.006
Sample - 4	0.001	0
Sample - 5	1.04	1.35

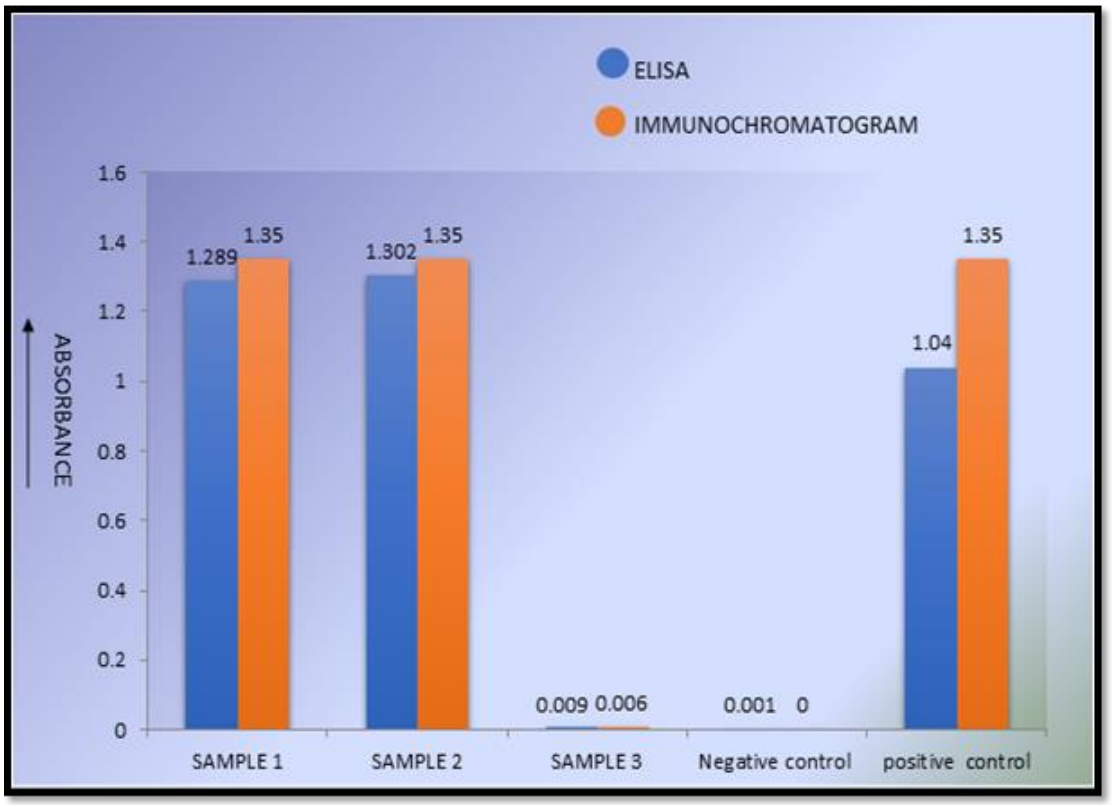

Fig 11. The accuracy relevance of immunochromatography and ELISA

CONCLUSION

The production of monoclonal antibodies (MAbs) against Hepatitis B surface antigen (HBsAg) led to the development of a pair of mAbs for a qualitative ELISA. In our sandwich ELISA kit, these monoclonal antibodies against HBsAg were utilized as both the capture and detection antibodies. The ELISA test system underwent validation using human serum samples that were either positive or negative for Hepatitis B.

There are numerous commercially available rapid immunochromatographic tests (ICTs) claiming high sensitivity and specificity for HBsAg detection. These tests are frequently used by peripheral labs and hospitals without conducting in-house validation, which can lead to performance variability. This variability can arise from differences in components such as conjugate pads and substrate membranes. ICTs tend to exhibit lower sensitivity and specificity, particularly with larger sample volumes.

Our evaluation of both rapid diagnostic tests and ELISA indicates that ELISA is more efficient than ICT. Therefore, ICTs should be recommended only for screening purposes in resource-limited areas and peripheral health facilities. Considering the high risk of Hepatitis B virus (HBV) infection within communities, false negative results pose a significant threat of silent transmission and disease spread. This underscores the need for more sensitive assays, such as ELISA, to ensure accurate diagnosis and control of HBV.

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