Development of Diagnostic Dip Strip Immunoassay Using Antibodies of PreS₂ Region of Hepatitis B Surface Antigen

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Abstract.- An immunoassay based on immunochromatographic lateral flow is developed for qualitative determination of HBsAg in serum samples using anti-preS₂ antibodies. The diagnostic test strip consists of four components i) sample application pad ii) conjugate pad iii) nitrocellulose membrane and iv) absorbent pad. Affinity purified anti-HBsAg antibodies and goat anti-rabbit IgG antibodies were coated onto nitrocellulose membrane as a test line and control line, respectively. The affinity purified anti-Pre-S₂ antibodies conjugated to colloidal gold particles (20 nm) were coated on polyester conjugate pad. The conjugate pad was attached to nitrocellulose membrane and the sample application pad was attached to conjugate pad. The absorbent pad comprising 3mm Whatman paper was attached to the nitrocellulose strip opposite to the sample pad. Purified HBsAg (1.4µg/µl in PBS), 100 µl, was loaded on sample pad and allowed to flow over the strip for 10 minutes. Pink colour test line indicates the correct binding of gold labeled antibodies to antigen. The staining of control line confirm s the accuracy of the strip. The sensitivity and specificity of dip strip was measured by using serum samples of one hundred clinically confirmed HBV positive individuals and twenty HBV negative individuals. The test line appeared colored in the case of HBV positive individuals and remained blank in the case of negative individuals. The results of indigenous dipstrip were compared with the results of imported dipstrips. The results indicated that indigenous dipstrip is as good as the imported one, therefore, recommended for use in clinical trials.

Key words: PreS₂ epitope, colloidal gold particles, dipstrip immunoassay, HBsAg.

INTRODUCTION

Hepatitis is an inflammation of the liver. It is usually caused by viral infections, toxic agents or drugs but may also be due to an autoimmune response (Shapiro, 1993). The different types of viral hepatitis are A (formerly called infectious hepatitis), B (serum hepatitis), C (formerly called non-A, non-B hepatitis), D (delta hepatitis), E (a virus transmitted through the faeces of an infected person), F and G, (cryptogenic caused by a virus as yet unidentified) (Hadmen et al., 2004). Hepatitis B is a viral infection of the liver caused by the Hepatitis B virus (HBV). Hepatitis B virus infections result in 500,000 to 1,200,000 deaths per year worldwide due to the complications of chronic hepatitis, cirrhosis and hepatocellular carcinoma (Szmuness, 1978; Chen et al., 2005). Hepatitis B endemic in a number of (mainly South-East Asian) countries, leading to cirrhosis and hepatocellular carcinoma (Hadmen et al., 2004). HBV is a double stranded, enveloped DNA virus of the Hepadnaviridae family, which replicates in the liver and causes hepatic dysfunction (Tiollais et al., 1985).

Hepatitis B surface antigen (HBsAg) is found on the surface of the virus that also appear in the blood as 22-nm spherical and tubular particles when produced in excessive quantity (Peterson, 1987; Kann and Gerlich, 1997). Hepatitis B surface antigen (HBsAg), the first serologic marker of HBV infection, can be detected from 2 to 12 weeks after infection with HBV (Lee and Vyas, 1987). There are three immunoreactive epitopes (S, pre-S₁ and pre-S₂) on the surface of HBsAg (Peterson, 1987). There are numerous serological procedures available for the detection of HBsAg (McCready et al., 1991; Kane et al., 1988). Dip strip immunoassay is the one of those serological assays. This assay is
an inexpensive, disposable, membrane based, quick
and provides visual evidence of the presence of an
analyte in the liquid sample (Lucocq, 1992). The
base substrate of this immunoassay is typically a
nitrocellulose strip onto which capture binding
protein, usually antibody or antigen, is adsorbed as
test and as a control line (Chandler et al., 2000).
Another conjugate pad attached to the nitrocellulose
membrane strip that contains dried gold particles
adsorbed with antibodies or antigens that may
interact with analyte being detected. The sample pad
onto which sample is loaded is attached to the
conjugate pad. The sample migrates by capillary
diffusion through the conjugate pad allowing the
interaction of the sample analyte with the gold
conjugated antibodies. The complex of gold
conjugate and the analyte then moves onto the
membrane strip. After reaching the capture binding
protein, it becomes immobilized and produces a
distinct signal in the form of a sharp red line. A
second line, the control, is also formed on the
membrane by excess gold conjugate, indicating the
accuracy of the test (Chandler et al., 2000).

In this study, an indigenous dip strip immunoassay using anti-PreS2 epitope which is
never used before for the qualitative determination
of hepatitis B surface antigen (HBsAg) from serum
or whole blood of HBV infected individuals has
been developed.

MATERIALS AND METHODS

All chemicals used in this study were
purchased from Sigma.

Characterization of antibodies in immunized serum
Antibodies produced in rabbit against
hepatitis B surface antigen and synthetic pre-S2
peptide were checked by immunodot blot and
ELISA and purified as described by Samra et al.
2006. The concentration of purified antibodies was
determined as described by Bradford (1976) using
human IgG as standard.

Preparation of colloidal gold particles
All glassware used for gold particles
preparation was siliconized with siliconizing agent.
Colloidal gold particles (20 nm) were prepared by
the reduction of chloroauric acid as described by
Walker (1994). Briefly, Fifty milligrams of
chloroauric acid was dissolved in 500ml of doubled
distilled deionized water and the solution was boiled
under vigorous stirring, 12.5 ml of 1% tri-sodium
citrate was added. The solution turned from a
slightly yellow to a dark blue and finally to an
orange-red color. Then solution was cooled down to
room temperature. The pH was adjusted 7.5 by
addition of 0.1 M K2CO3 and stored at 4°C.

Conjugation of PreS2 antibody to gold particles
The anti preS2 antibodies was conjugated to
gold particles as described by Romano et al. (1974)
and Faulk and Taylor (1979). Briefly, 100 μl of anti-
preS2 antibody was rapidly added to 1ml of gold
solution and stirred for 30 min at 25°C and
centrifuged at 10,000 rpm for 15 minutes at 4°C.
The pellet was separated and resuspended in 5ml of
stabilizing buffer (0.15 M sodium chloride, 50mM
Tris-Cl, pH 7.4, 0.5mg/ml polyethylene glycol-4000
and 0.001% sodium azide). The binding of gold
particles to antibodies was confirmed by
flocculation test, immunodot blot and western
blotting (Walker, 1994).

Characterization of anti-preS2 antibody-gold
complex
Immunodot blot analysis
Immunodot blot analysis was done as
described by Walker (1994) and Hus (1984) with
little modifications. Nitrocellulose membrane was
cut in 3 x 5cm then 10μl of purified hepatitis B
surface antigen was spotted on the membrane and
was allowed to dry. The nitrocellulose membrane
was stained with acidic Ponceau S, for 5 minutes in
order to check the presence of protein. The
membrane was destained with 1xTBS. The
membrane was dipped in blocking buffer (3 % BSA in TBS) for 45 minutes at
37°C to block non specific binding. After 5-6
washing with 1xTBS, membrane was dipped in anti-
preS2-gold complex (1:10 dilution with TBS) for 45
min with constant shaking and results were noted.

SDS-PAGE and western blot
Purified HBsAg was run on 10% SDS-PAGE
as described by Laemmli (1970) and the protein was
transferred onto nitrocellulose membrane as described by Towbin et al. (1979) in Invitrogen XCell™ Blot Module. The immunoglod staining for denatured HBsAg was done in similar fashion to immunodot blot analysis.

Preparation of dip strip

Nitrocellulose membrane was cut into 3.0 cm x 0.3 cm strip. 3µl of purified anti-HBsAg antibody (0.1 μg / ml) was spotted on the membrane 1.0 cm above the bottom of the paper as the test line. Whereas, 3µl of goat anti-rabbit IgG (0.1 μg / ml) was spotted as a control line 1.0 cm above the test line. The membrane was allowed to air dry for 45 minutes at 4°C and then blocked by incubating in mixture 3% bovine serum albumin and 2% gelatin in TBS for 30 minutes. The conjugate pad was a strip of 0.8 cm x 0.3 cm of polyester sheet. 100µl of gold labeled anti-pre-S2 antibody was loaded on to the conjugate pad and dried at 4°C. The conjugate pad was attached at the bottom of nitrocellulose membrane. A sample application pad of 0.5cm x 0.3 cm strip of polyester sheet was attached onto the conjugate pad. A strip of 0.5cm x 0.3cm of 3mm Whatman paper was attached as absorbent pad to the end of membrane opposite to the conjugate pad. This strip was laminated on a plastic strip (3.0 cm x 0.3 cm) and stored at 4°C. The construct of dipstrip is shown in Figure 1.

![Fig. 1. Indigenous dip strip.](image)

Evaluation of dip strip with purified HBsAg

To determine the accuracy of indigenous dip strip, it was evaluated with purified hepatitis B surface antigen. 100µl of purified HBsAg (1.5µg/µl) was added on sample application pad of dip strip. The sample was allowed to flow on strip for 10 minutes and noted the results.

Evaluation of dip strip using serum sample of HBV positive and negative individuals

Blood samples from one hundred clinically investigated HBV positive individuals and twenty HBV negative individuals were collected. Serum was isolated by standard procedure (Harlow, 1988). 100µl of serum of HBV positive and negative individuals were loaded on sample application pad of separate dip strip and allowed to flow for 10 minutes. After 10 minutes test results were observed. The collected sera samples were also processed for inactivation of hepatitis B virus. Each sample was mixed with 100µl of 0.05M Tris–Cl pH, 7.5 and 100µl of 1% SDS and was incubated at 60°C for 1.5 hour. After incubation, the samples were centrifuged at 10,000 rpm for 10 minutes. The supernatants were separated and stored at – 20°C or processed for ELISA.

Comparison of indigenous dip strip with the dip strip commercially available in the market

To determine the sensitivity and specificity of indigenous dip strip, positive and negative HBV serum samples were separately analyzed on indigenous and commercially available dip strip.

ELISA of serum samples

100µl (1:100 dilution in TBS) of each inactivated serum sample and control serum sample was coated on microtitre plates and proceeded as described by Samra et al. (2006).

RESULTS AND DISCUSSION

Detection of anti-preS2 and anti-HBsAg antibodies in immunized serum

The immunized serum was analyzed for the presence of anti-PreS2 and anti-HBsAg antibodies by immunodot blot and ELISA. The appearance of purple blue spot on nitrocellulose membrane in immunodot blot analysis and appearance of yellow
colour in ELISA (Enzyme linked immunosorbant assay) indicated the presence of anti-pre-S2 antibodies and anti-HBsAg antibodies in immunized serum. The activity on nitrocellulose membrane is shown in Figure 2.

![Image A](image1.png) ![Image B](image2.png)

**Fig. 2.** Immunodot blot screening of antibodies; A, immunized serum showing the presence of anti-pre-S2 antibodies; B, immunized serum showing the presence of anti-HBsAg antibodies.

Characterization of gold labeled anti-preS2 antibodies

Gold labeled anti-pre-S2 antibodies were characterized using immunodot blot and western blot. Pink colour was deposited on the nitro cellulose membrane which indicated that gold labeled anti-pre-S2 antibodies are specific to pre-S2 epitope of HBsAg (Fig.3).

![Image A](image3.png) ![Image B1](image4.png) ![Image B2](image5.png)

**Fig. 3.** Gold labeled anti-Pre-S2 antibodies assays; A, Immunodot blot analysis using gold labeled anti-preS2 antibodies; B, western blot analysis (B2) indicating the binding of HBsAg with gold labeled anti-preS2 antibodies and (B1) Control strip did not show any binding.

Evaluation of dip strip

The strip was pre-evaluated using purified HBsAg as sample. The appearance of spots on both test region and control region indicated the presence of HBsAg in the sample and accuracy of dip strip (Fig.4).

In case of HBV positive individuals the spots appeared on both test and control region indicating that the serum sample contain HBsAg (Fig.4). While in case of HBV negative individual the spot appeared on control region only which demonstrate that the serum sample did not contain HBsAg (Fig.4).

![Image A](image6.png) ![Image B](image7.png)

**Fig. 4.** Dipstrip immunoassays, (C) Control line, (T) Test line: A, Both test and control lines indicating the presence of purified HBsAg; B, Both test and control line indicating the presence of HBsAg in serum sample; C, Staining on control line only indicating the absence of HBsAg in serum sample.

Comparison of indigenous dip strip with ELISA and commercial dip strip using HBV positive and negative serum samples

One hundred serum samples from clinically investigated HBV positive individual and 20 samples from HBV negative individuals were analyzed using indigenous dip strip, ELISA and commercial dip strip. All serum samples positive in indigenous dip strip were found positive when analyzed with ELISA and commercial dip strip. The HBV negative serum samples were negative in ELISA, indigenous and commercial dip strip which indicates that dip strip results are comparable with ELISA in terms of sensitivity and specificity.

Amongst the serological immunoassays available for the detection of HBsAg, the first serological marker of HBV infection, such as
ELISA, indirect immunofluorescence assay are very common (Graczyk, 1996). These assays cannot be easily applied in the field because of their special requirement in term of equipment, chemicals and cumbersome nature. In the past different types of modifications has been developed in ELISA to make it more field applicable but the problem is not yet solved (Suga et al., 2000). Later on another assay called immunodot blot was introduced where nitrocellulose membrane is used as a test matrix (Peppas et al., 1983; Scott, 1983). In immunodot blot assay colloidal gold labeled antibodies are used for detection of antigen instead of enzyme labelled antibodies (Moremans, 1984; Hus, 1984). To further make it more field test friendly, another assay called dip strip assay was developed for the detection of HBsAg from human serum (Delmulle et al., 2005; Graczyk, 1996; George, 2002; Lau et al., 2003). In this assay HBsAg antibodies are used for the detection of HBsAg.

In order to make this test more precise and accurate the anti preS2 antibodies are used to prepare indigenous dip strip instead of anti HBsAg antibodies that are used in commercial dip strip. The results obtained from the indigenous dip strip are comparable with the results obtained of ELISA as well as the results of commercial dip strip assay.

The indigenous dip strip can be used for screening of population for hepatitis B virus infection in our local population because it is inexpensive field friendly and does not requires any instrument.

REFERENCES


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