Pakistan J. Zool., vol. 39(3), pp. 185-190, 2007.

Development of Diagnostic Dip Strip Immunoassay Using Antibodies of $PreS_2$ Region of Hepatitis B Surface Antigen

ZAHOOR QADIR SAMRA, MUHAMMAD SHAHBAZ ASLAM, HINA SHAUKAT, NADIA DAR, AND MUHAMMAD AMIN ATHAR

Institute of Biochemistry and Biotechnology, Quaid-i-Azam Campus, University of the Punjab, Lahore-54590 (ZQS, MSA, HS, MAA) and Jinnah Degree College for Women, Mozang Road, Lahore (ND)

Abstract.- An immunoassay based on immunochromatographic lateral flow is developed for qualitative determination of HBsAg in serum samples using anti-$preS_2$ 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-S_2 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 confirms 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: $PreS_2$ 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-S_1 and pre-S_2) 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

0030-9923/2007/0003-0185 $ 8.00/0

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-PreS_2 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-S_2 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 silconizing 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 K_2CO_3 and stored at 4^{o}C.

Conjugation of PreS_2 antibody to gold particles

The anti preS_2 antibodies was conjugated to gold particles as described by Romano *et al*. (1974) and Faulk and Taylor (1979). Briefly, 100 µl of anti-preS_2 antibody was rapidly added to 1ml of gold solution and stirred for 30 min at 25^{o}C and centrifuged at 10,000 rpm for 15 minutes at 4^{o}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 polyethyleneglycol-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

Immunodotblot analysis

Immunodotblot 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 1xTris-buffered-saline (TBS). 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-preS_2-glod 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-S_2 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.

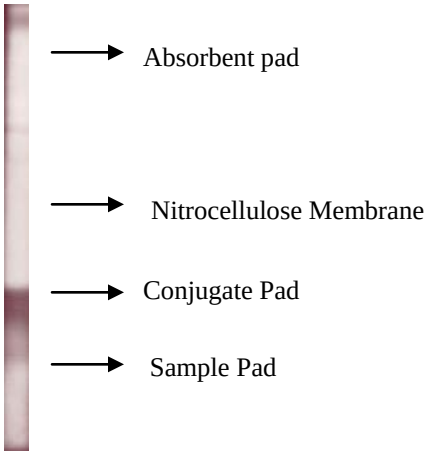

Fig. 1. Indigenous dip strip.

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-preS_2 and anti-HBsAg antibodies in immunized serum

The immunized serum was analyzed for the presence of anti-PreS_2 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-S_2 antibodies and anti-HBsAg antibodies in immunized serum. The activity on nitrocellulose membrane is shown in Figure 2.

Fig. 2. Immunodot blot screening of antibodies; A, immunized serum showing the presence of anti-pre-S_2 antibodies; B, immunized serum showing the presence of anti-HBsAg antibodies.

Characterization of gold labeled anti-preS_2 antibodies

Gold labeled anti-pre-S_2 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-S_2 antibodies are specific to pre-S_2 epitope of HBsAg (Fig.3).

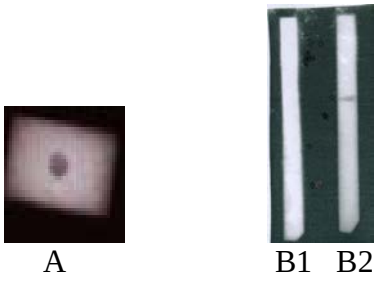

Fig. 3. Gold labeled anti-Pre-S_2 antibodies assays; A, Immunodot blot analysis using gold labeled anti-preS_2 antibodies; B, western blot analysis (B2) indicating the binding of HBsAg with gold labeled anti-preS_2 antibodies and (B1) Control strip did not show any binding.

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).

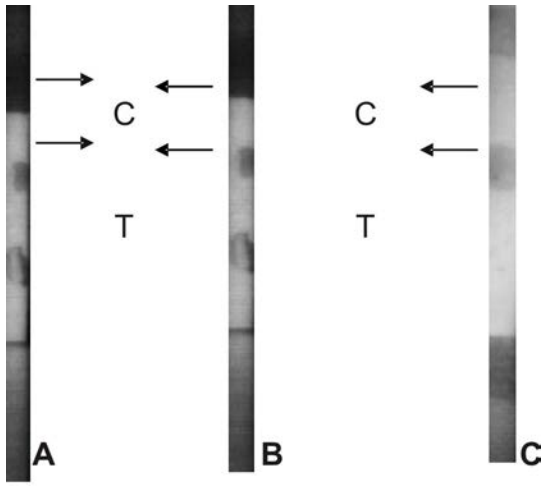

Fig. 4. Dipstrip immunoassays, (C) Control line, (T) Test line: A, Both test and control lines indicating the presence of purified HBsAg; B, Both test and control line indicating the presence of HBsAg in serum sample; C, Staining on control line only indicating the absence of HBsAg in serum sample

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 $preS_2$ 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

BRADFORD, M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Ann. Biochem*., **72**: 248-254

CHANDLER, J., TRACEY, G. AND NICOLA, R., 2000. *Assay device and method*, European patent application: 96901447.1.

CHEN, C.Y., WU, J.R., ZHU, X.Y. AND WANG, S.Y., 2005. Hepatitis B virus infection. *World J. Gastroenterol*., **13**:873-875.

DELMULLE, B.S., SAEGER, S.M.D.E, SIBANDA, L., BARNA-VETRO, I. AND PETEGHEN, C.H., 2005. Development of an immunoassay based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed. *J. Agric. Fd. Chem.*, **53**:3364-3368.

FAULK, W. AND TAYLOR, G., 1979. An immunocolloidal method for the electron microscopy. *Immunochemistry*, **8**: 1081-1083.

GEORGE, M., 2002. Immunochromatographic strip test for rapid detection of diphtheria toxin. *J. clin. Microbiol.*, **40**: 80-83.

GRACZYK, T.K., CRANFIELD, M.R. AND FAYER, R., 1996. Evaluation of commercial enzyme immunoassay (EIA) and immunofluorescent antibody (IFA) test kits for detection of cryptosporidium oocysts of species other than cryptosporidium parvum. *Am. J. trop. Med. Hyg*., **54**: 274-279.

HADMEN, J., WEDEMEYER, H. AND MANNS, M.P., 2004. Hepatitis as a travel disease. *Internist*, **45**: 655-668.

HARLOW, E.D. AND LANE, D., 1988. *Antibodies, A laboratory manual*. Cold Spring Harbour Labs., USA.

HUS, Y., 1984. Immunogold for detection of antigen on nitrocellulose paper. *Anal. Biochem*., **142**: 221-225

KANE, M.A, HADLER, S.C. AND MARGOLIS, H.S., 1988. Routine prenatal screening for hepatitis B surface antigen. *J. Am. med. Assoc*., **259**:408-409.

KANN, M. AND GERLICH, W.H., 1997. Replication of hepatitis B virus. *Intervirology*, **12**: 63–116.

LAEMMLI, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

LAU, D.T.Y., LEMON, H.M.M., DOO, E., GHANY, M.G., MISKOVSKY, E., WOODS, G.L., PARK, Y. AND HOOFNAGLE, J.M., 2003. A rapid immunochromatographic assay for hepatitis B virus screening. *J. Viral Hepatitis*, **10**: 331-335.

LEE, H.S. AND VYAS, G.N., 1987. Diagnosis of viral hepatitis. *J. clin. Microbiol*., **7**: 741-57.

LUCOCQ, J., 1992. Quantitation of gold labeling and estimation of labeling efficiency with a stereological counting method. *J. Histol. Cytochem*., **40**: 1929-1935.

MCCREADY, L.A., MORENS, D. AND FIELDS, H.A., 1991. Evaluation of EIA as a screening method for hepatitis B marker in an open population. *Epidemiol. Infect.*, **107**: 673-684.

MOEREMANS, M., DANEELS, G. AND VANDIJCK, A., 1984. Sensitive visualization of antigen-antibody reactions in dot and blot immune overlay assays with immunogold and immunogold/silver staining. *J. Immunol. Methods*, **74**: 353-360.

PEPPAS, M., HAJKOWSKI, R. AND HOCKMEYER, W., 1983. Dot enzyme-linked immunosorbent assay (dot-ELISA): a micro technique for the rapid diagnosis of visceral leishmaniasis. *J. Immunol. Methods*, **64**: 205-214.

PETERSON, L.D., 1987. The structure of hepatitis B surface antigen and its antigenic sites. *Bioessays*, **6**:258-262.

ROMANO, E.L., STOLONSKI, C. AND HUGHES-JONES, N.C., 1974. An immunoglobulin reagent labeled with

colloidal gold for use in electron microscopy. *Immunocytochemistry*, **14**:711-715.

SAMRA, Z.Q., SIDDIQUE, Z., DAR, N. AND ATHAR, M.N., 2006. Immunochemical detection of pres2 epitope of hepatitis B surface antigen. *Pakistan. J. Zool.*, **38:** 291-296.

SCOTT, D., 1983. Immunoblotting and dot blotting. *J. Immunol. Methods*, **119**:153-156.

SHAPIRO, C. N., 1993. Epidemiology of hepatitis B. *Pediat. Infect. Dis. J*., **12**: 433-437.

SUGA, M., ARIMA, K. AND YACHI, A., 2000. Detection of IgM, IgA and IgG antibodies to preS2 antigen in HBV infection. *Digestion*, **50**: 153-61.

SZMUNESS, W., 1978. Hepatocellular carcinoma and the hepatitis B virus. Evidence for a casual association. *Prog. Med. Virol*., **32**:264-268.

TIOLLAIS, P., POURCEL, C. AND DEJANS, A., 1985. The hepatitis B virus. *Nature*, **317**: 489-495.

TOWBIN, H.K., STAEHELIN, T. AND GORDON, J., 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. natl. Acad. Sci.*, USA, **76**: 4350-4359.

WALKER, J. M., 1994. The detection of proteins on blots using gold or immunogold. In: *Methods in molecular biology, basic protein and peptide protocol*, vol. 32, pp. 239-255. Humana Press Inc, Totowa, NJ.

(Received 16 February 2007, revised 15 May 2007)