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Development of multiplex hepatitis B test strip for hepatitis B screening

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ABSTRACT

Clinical diagnosis of hepatitis B virus (HBV) infection can be detected from presence of serological markers of HBV infection including hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs) and hepatitis B core antibody (anti-HBc), of which different combinations are used to identify the different stages of the disease. Typical diagnostic test utilizes enzyme immunoassay (EIA) technique, which takes a long time and requires elaborate equipment for analysis. This study purposes a technique that allows simple testing with shorter test time thus can provide easy access for the patients. In this study, we present a multiplex hepatitis B test strip for hepatitis B identification. The fabricated multiplex hepatitis B test strip can be used for simultaneous detection of 3 serological markers, i.e., HBsAg, anti-HBs and anti-HBc. The assay is based on principle of sandwich immunochromatographic assay (ICA) for HBsAg detection while, for anti-HBs and anti-HBc detections, the assay is based on indirect ICA. Gold nanoparticles based ICA was used for signal generation. The assay time was less than 15 minutes which is suitable for rapid on-site testing. The developed multiplex hepatitis B test strip showed good result for anti-HBc test but showed low sensitivity for HBsAg and anti-HBs test. However, the developed hepatitis B test strip is selective and there is no cross-reaction between each serological marker. Therefore, this work can be a good prototype for multiplex hepatitis B test strip development to fabricate more effective multiplex hepatitis B test strip in the future.

1. INTRODUCTION

Hepatitis refers to the inflammatory condition of the liver, which can cause liver malfunction. The leading cause of hepatitis is hepatitis B virus (HBV) infection. Besides hepatitis, HBV can also cause cirrhosis and liver cancer (Randrianirina, 2008). There are about 240 million people chronically infected with HBV (hepatitis B) surface antigen presents for at least 6 months) and more than 686,000 people die every year due to complications of hepatitis B (World Health Organization, 2016). Therefore, ideally,

everyone should be screened for HBV to control transmission and obtain proper treatment in case of infection. In clinical diagnosis, HBV infection can be detected from serological markers including hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs) and hepatitis B core antibody (anti-HBc), which vary in each phase of infection (Krajden, 2005). Conventional method for hepatitis B serological markers detection is enzyme immunoassay (EIA) (McCready, 1991; Sato, 1996; Shen, 2015). Although this method is highly sensitive, reliable and widely used but it requires complicated equipment, well-trained personnel and long reaction time. In addition, traditional method for HBV screening poses high cost for diagnosis that makes it not easily accessible to people in some developing countries. Therefore, immunochromatographic assay (ICA) was proposed to resolve these problems.

ICA is a popular point-of-care (POC) device because it is simple, rapid, cost effective and easy to use for end user (Dzantiev, 2014). There are many manufacturers producing immunochromatographic test strip for hepatitis B's serological markers including HBsAg, anti-HBs and anti-HBc. Rapid tests for each marker were evaluated compare to conventional method. For example, Determine^{TM} HBsAg rapid immunochromatographic test (Abbott Laboratories, Wiesbaden, Germany), Virucheck® HBsAg (Orchid Biomedical Systems, Goa, India), Cypress HBsAg Dipstick® (Cypress Diagnostics, Langdorp, Belgium) and Hexagon® HBsAg (Human, Wiesbaden, Germany) were evaluated by Randrianirina and coworkers. All of four HBsAg rapid tests show sensitivity and positive predictive value (PPV) exceeded 95%, and specificity and negative predictive value (NPV) exceeded 96% (Randrianirina, 2008). The HBsAg One Step Hepatitis B Surface Antigen Test Device and the HBsAb One Step Hepatitis B Surface Antibody Test Device (General Biologicals Corporation, Hsinchu, Taiwan) were checked by Fu-Yu Wu and coworkers. HBsAg test card showed that the sensitivity was 88.8% and the specificity was 100%, while anti-HBs test card presented the sensitivity as 91.8% and the specificity as 96.5% (Wu, 2016). El-Ghitany and coworkers evaluated non HBsAg test cards, i.e. anti-HBs, anti-HBc and anti-HBe from Intec Products, Fujian of China. They reported sensitivity of all tests as 64.2%, 85.48%, and 82.78% respectively (El-Ghitany, 2013). Although available test strips show high sensitivity for each marker but manufacturer want to make diagnosis easier. For easier in screening, multi HBV test cards were produced, for example, OnSite HBV 5-Parameter Rapid Test from CTK Biotech, Inc. However, even though this multi-test card can detect simultaneous five serological markers but the principle is based on one marker per one strip and all of strips were assembled in one plastic cassette. Therefore, there is no difference between multi test and individual test. In addition, some of the markers are not essential for screening causing some tests to be wasteful. Moreover, in case of limited serum or plasma sample, dropping serum or plasma sample to five positions is difficult. To solve these problems, we present a multiplex hepatitis B test strip for hepatitis B identification which can detect simultaneous 3 essential serological markers, i.e., HBsAg, anti-HBs and anti-HBc. The developed multiplex HBV test strip was tested with recombinant HBsAg protein and antibody (anti-HBs and anti-HBc) from mouse as a model to evaluate it's sensitivity and specificity.

2. MATERIALS AND METHODS

2.1 Chemicals and materials

Recombinant hepatitis B surface antigen ad protein (ab193473), anti-hepatitis B virus surface antigen antibody [hb6] (ab38733), anti-hepatitis B virus surface antigen antibody [hb12] (ab2039), recombinant hepatitis B virus core antigen protein (ab49013), anti-hepatitis B virus core antigen antibody [C1] (ab18683), goat anti-mouse IgG H&L (ab6708), rabbit anti-mouse IgG H&L (ab6709) and mouse monoclonal 2A9 anti-rabbit IgG heavy chain (ab99699) were purchased from Abcam. Nitrocellulose (NC) membrane glass fiber and plastic backing card were purchased from Serve science company, Thailand. Cellulose fiber was purchased from Merck Millipore. Gold nanoparticles (GNPs) 40 nm size stabilized in 0.1 mM PBS were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Bio basic Canada inc. All of antigens and antibodies were diluted in 0.01 M phosphate buffer solution (PBS) pH 7.4.

2.2 Conjugation of GNPs to anti-hepatitis B virus surface antigen antibody and rabbit anti-mouse IgG

The GNPs conjugated antibodies were prepared as described previously (Ang, 2016) with slight modification. The 40 nm GNPs were adjusted to pH 7.0 by 0.2 M K_2CO_3 then anti-hepatitis B virus surface antigen antibody (ab38733) was added to 10 µg/mL final concentration. Rabbit anti-mouse IgG (ab6709) was conjugated with GNPs using the same procedure. The mixed solutions were gently shaken at room temperature (RT) for 1 hour. BSA was added to 1%w/v final concentration and mixed for 1 hour at RT with gentle shaking. The mixture was centrifuged at 4,000 g for 30 minutes at 4ºC and supernatant was discarded. The pellet of GNPs conjugated antibodies were resuspended in 0.01 M PBS containing 1%w/v BSA. The GNPs conjugated antibodies were stored at 4ºC until use.

2.3 Characterization of GNPs conjugated antibodies

Both of conjugated components were characterized by absorbance scanning compared with bare GNPs. Both of GNPs conjugated antibodies were diluted in PBS at 1:10 ratio. 100 µL of bare GNPs, diluted GNPs conjugated anti-HBs and diluted GNPs conjugated rabbit anti-mouse antibodies were added to 96 well plate and measured absorbance scan at 450 to 650 nm using TECAN microplate reader model infinite M 200 and I-control software.

2.4 Fabrication of the multiplex hepatitis B test strip

The multiplex hepatitis B test strip developed here consists of a sample pad, conjugate pads, NC membranes and absorbent pads. The fabricated test strip was divided into 2 sides, the first side was for HBsAg test and the other side was for anti-HBs and anti-HBc tests. Anti-hepatitis B virus surface antigen antibody [hb12] (ab2039) was coated as a test line, while goat anti-mouse IgG H&L (ab6708) was coated as a

control line on the NC membrane. Another sheet of NC membrane was coated with HBsAg and HBcAg as test lines while mouse monoclonal 2A9 anti-rabbit antibody (ab99699) was coated as a control line. Both sheets of NC membrane were dried at RT until completely dry. All membrane was then blocked with 1%w/v BSA in 0.01 M PBS for 1 hour. After that, NC membranes were washed with 0.01 M PBS 3 times and left out to dry at RT. For conjugate pad, glass fiber was soaked in 0.01 M PBS containing 1%v/v TWEEN 20, 2%w/v BSA and 2.5%w/v sucrose for 30 minutes and dried at RT. One sheet of conjugate pad was saturated with GNPs conjugated anti-hepatitis B virus surface antigen antibody and the other sheet was saturated with GNPs conjugated rabbit anti-mouse IgG. Both of conjugate pads were dried at 37ºC for 1 hour and stored at 4ºC until use. Sample pad was pretreated using the same procedure used for the conjugate pad. The sample pad, conjugate pads, immobilized NC membranes and absorbent pads were overlaid on plastic backing card with 2-mm overlap between each component. All prepared strips were cut into 0.5-cm width to fabricate multiplex hepatitis B test strips. Schematic of the developed multiplex hepatitis B test strip is shown in fig. 1.

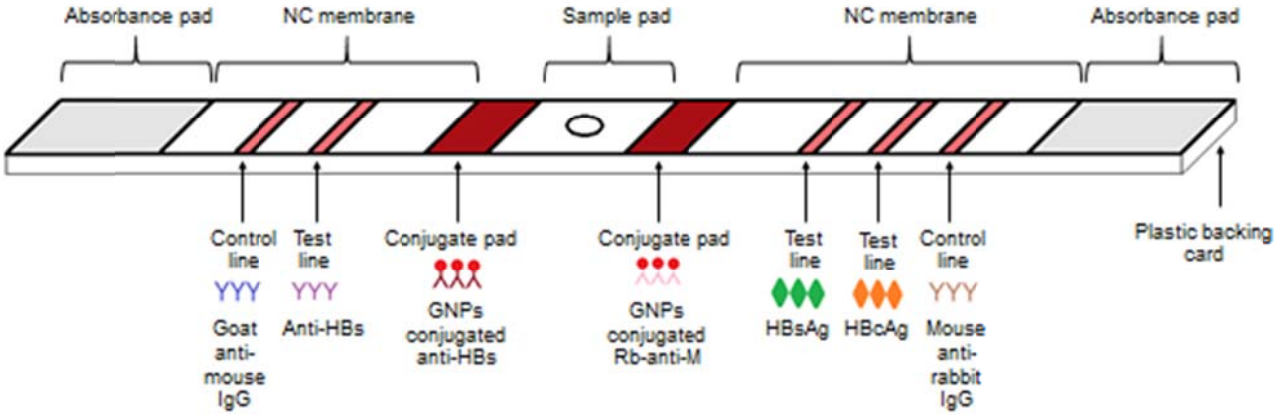

Fig. 1**.** Schematic of the developed multiplex hepatitis B test strip and immobilization of antigens and antibodies on strip.

2.5 Detection strategy of the multiplex hepatitis B test strip

The detection strategy of the multiplex hepatitis B test strip is based on sandwich immunoassay and indirect immunoassay as shown in fig.2. Briefly, 200 µL of sample was added to sample pad on the center of strip. If only HBsAg is present in the sample, HBsAg will bind with GNPs conjugated anti-HBs to form GNPs-anti-HBs-HBsAg complex and then the immunocomplex will be captured by another anti-HBs to form test line. Excess GNPs conjugated anti-HBs will be captured by goat anti-mouse antibodies to form control line. In contrast, the other side of the strip will show signal only on the control line from immunocomplex between GNPs conjugated rabbit anti-mouse IgG and mouse anti-rabbit antibody.

In case of anti-HBs presents in the sample, anti-HBs will bind with GNPs conjugated rabbit anti-mouse IgG to form GNPs-rabbit anti-mouse IgG-anti-HBs complex and then

the immunocomplex will be captured by HBsAg at the test line. Excess GNPs conjugated rabbit anti-mouse IgG would be captured by mouse anti-rabbit antibody to form control line. In contrast, another side of strip would show signal only control line from immunocomplex between GNPs conjugated anti-HBs and goat anti-mouse antibody. If anti-HBc is present in the sample, developed immunosensor will work based on the similar principle of anti-HBs test but differ in the captured antigen, signal will be presented at test line which was coated by HBcAg.

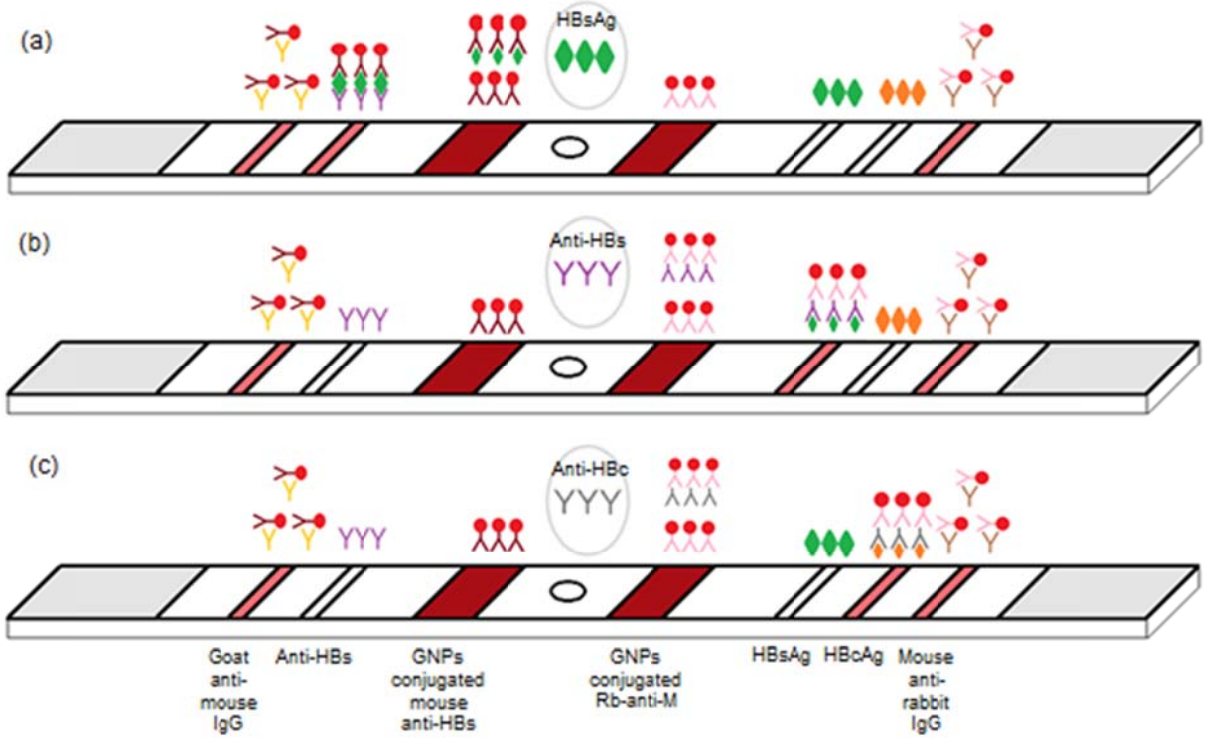

Fig. 2. Detection strategy of the developed multiplex hepatitis B test strip. (a), (b) and (c) are HBsAg, anti-HBs and anti-HBc test respectively.

3. RESULTS AND DISCUSSION

3.1 Characterization of GNPs conjugated antibodies

As the signal was generated by aggregation of GNPs at test and control lines, we need to ensure that antibodies used to capture analytes were immobilized on surface of GNPs. Both of GNPs conjugated antibodies were analyzed by UV/Vis spectrometry and compared with the bare GNPS as shown in fig. 3. From the spectra we can observe that the peak positions of GNPs conjugated anti-HBs and GNPs conjugated Rb-anti-M shifted from 530 nm to 534 nm when compared with bare GNPs. Thus, we proved that antibodies were absorbed on the surface of GNPs.

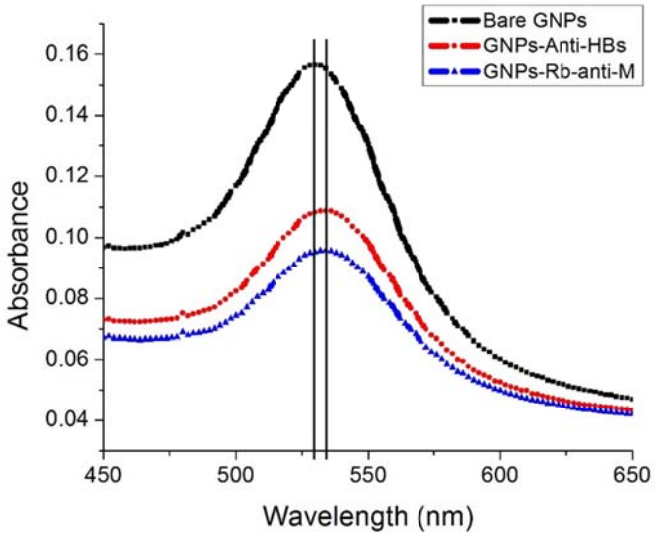

Fig. 3**.** Absorbance scans of GNPs conjugated antibodies compared with bare GNPs.

3.2 Performance test of the developed multiplex hepatitis B test strip

To obtain performance and specificity, the developed multiplex hepatitis B test strips were divided into 4 groups for each serological marker test, i.e. HBsAg, anti-HBs, anti-HBc and negative control (PBS). The developed test strips were tested for each marker. 200 µL of each diluted marker (1 µg/ml) was added onto the sample pad of each group and allowed for antigen/antibody reaction to occur for 15 minutes. Signal was determined from visibility of red line on test and control lines by naked eye. The results indicated that signal was observed only test and control line of each test (fig. 4.) and no signals from other markers were observed among the group of markers tested. The developed test strip showed high sensitivity (dark red line) for anti-HBc test but showed low sensitivity (light red line) in the case of HBsAg and anti-HBs tests. However, there are 4 subtypes of HBsAg, i.e. adr, adw, ayr and ayw that differ in amino acid sequences as well as the protein conformation (Peterson, 1984). Due to differences in HBsAg's structure of each subtype, binding ability between HBsAg and anti-HBs can be effected, which maybe the cause of faint signal observed as we used only HBsAg subtype ad for validation. In addition, fountain pen was used to draw test and control lines therefore the amount of captured reagent of each line maybe uneven which could effect the intensity and evenness of test and control lines. Therefore, we concluded that our developed immunosensor is selective for each marker and there is no cross-reaction between each serological marker.

However, this research is the primary state of development. We did not perform the test with real samples from patients, we only tested with recombinant HBsAg protein and mouse antibodies. In addition, we plan to develop multiplex hepatitis B test strip for human from this prototype by changing some antibodies on the strip which are rabbit anti-mouse IgG and mouse anti-rabbit IgG antibodies to mouse anti-human IgG and

goat anti-mouse antibodies, respectively. However, there are still many factors that should be validated in the future. Therefore, we hope that our multiplex hepatitis B test strip will be a good prototype for fabrication of multiplex hepatitis B test strip for human sample.

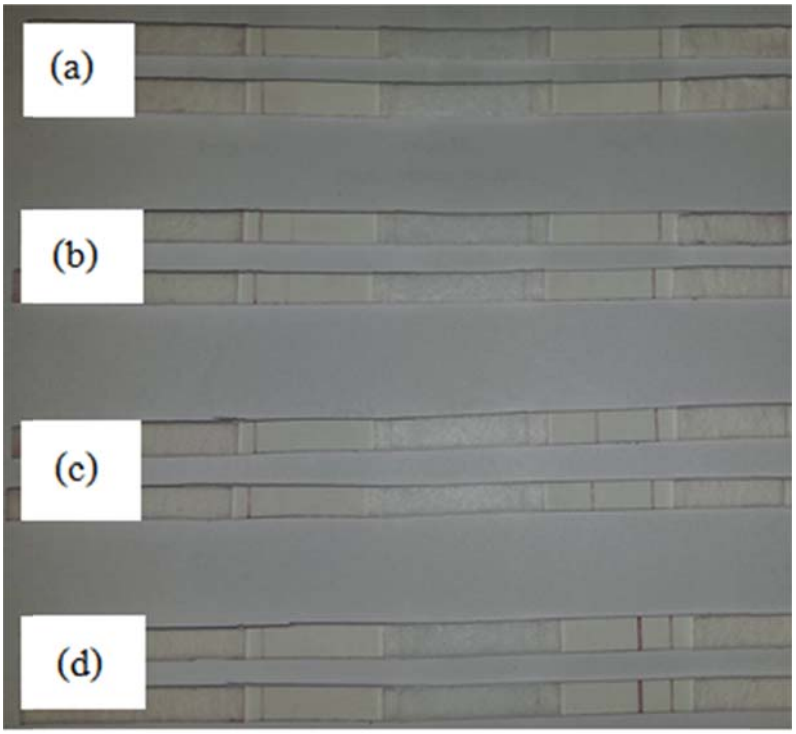

Fig. 4. The results of developed test strip when tested with (a) negative control (PBS), (b) HBsAg, (c) anti-HBs and (d) anti-HBc in duplicate.

4. CONCLUSIONS

In this research, we presented the idea of antigen and antibody arrangement for multiplex hepatitis B test strip fabrication. The results exhibited high sensitivity for anti-HBc test but showed low sensitivity for HBsAg and anti-HBs tests. Although our fabricated immunosensor showed low sensitivity but it showed great selectivity for each hepatitis B's marker. Therefore, our designed Ag/Ab arrangement can be used as a prototype for multiplex hepatitis B test strip fabrication in the future. As described above, this study is only at the primary state of development thus there are many issues that can be improved to enhance the effectiveness of the multiplex hepatitis B test strip fabrication.

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