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Development of One-Step Enzyme Linked Immunosorbent Assay for Hepatitis B Surface Antigen Utilizing Monoclonal Antibodies

การพัฒนาวิธีเอนไซม์อิมมูโนแอสเสย์ขั้นตอนเดียวเพื่อตรวจหาแอนติเจนชนิดผิวของไวรัสตับอักเสบบีโดยใช้โมโนโคลนอลแอนติบอดี

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การพัฒนาวิธีเอนไซม์อิมมูโนแอสเสย์ขั้นตอนเดียว (one-step ELISA) เพื่อตรวจหาแอนติเจนชนิดผิวของไวรัสตับอักเสบบี (HBsAg) ในซีรัม ทำโดยใช้โมโนโคลนอลแอนติบอดี (monoclonal antibody) ที่จำเพาะต่อ "a" determinant ซึ่งผลิตโดยการเชื่อมเซลล์มัยอีโลมา (P3-X63-Ag 8.653) กับเซลล์ม้ามของหนู BALB/c ซึ่งฉีดกระตุ้นด้วยวัคซีนไวรัสตับอักเสบบีสายพันธุ์ย่อย (subtype) adr และเปรียบเทียบผลการตรวจโดยวิธีที่พัฒนาขึ้นนี้กับวิธี ELISA ของน้ำยาตรวจสำเร็จรูป Sanofi Pasteur โดยทดสอบกับซีรัมจำนวน 447 ราย ที่ทราบผลการตรวจ HBsAg ด้วยน้ำยาตรวจสำเร็จรูป Abbott แล้ว (ผลบวก 245 ราย ผลลบ 202 ราย) วิธีที่พัฒนาขึ้นตรวจพบผลบวก 233 ราย ขณะที่วิธี ELISA ของน้ำยาตรวจสำเร็จรูปตรวจพบ 245 ราย การตรวจหา HBsAg โดยวิธีที่พัฒนาขึ้นมีความไวร้อยละ 95.1 ความจำเพาะร้อยละ 100 และความถูกต้องร้อยละ 97.3 โดยมีค่าการคาดคะเนบวกร้อยละ 100 และมีค่าการคาดคะเนลบร้อยละ 94.4 เมื่อเทียบกับน้ำยาสำเร็จรูป Sanofi Pasteur วิธีที่พัฒนาขึ้น ใช้โมโนโคลนอลแอนติบอดีที่มีความจำเพาะสูง และลดขั้นตอนการวิเคราะห์เป็นการวิเคราะห์แบบขั้นตอนเดียว จึงมีประโยชน์เหมาะสำหรับการตรวจตัวอย่างจำนวนมาก และทำให้ตรวจได้รวดเร็ว นอกจากนี้ยังใช้เป็นแนวทางในการพัฒนาวิธีการตรวจหาแอนติเจนชนิดอื่นได้

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Abstract : Nongluk Buddhirakkul, Prayute Buddhirakkul, Kruavon Balachandra and Suttichoke Jongtrakulsiri. 2000. Development of one-step enzyme linked immunosorbent assay for hepatitis B surface antigen utilizing monoclonal antibodies. Thai J Hlth Resch 14(1) : 19-26.

One-step ELISA assay using monoclonal antibody for screening of Hepatitis B surface antigen (HBsAg) was developed in our laboratory and compared with commercial ELISA test kit. Monoclonal antibody raised against the common "a" determinant of HBsAg was established by fusion of myeloma cell line (P3-X63-Ag 8.653) with spleen cells of BALB/c mice immunized with plasma derived hepatitis B virus vaccine (adr subtype) and used as probe to develop the one-step ELISA assay. Four hundred forty seven human sera (245 HBsAg-positive and 202 HBsAg-negative), previously tested for the presence of HBsAg by Abbott diagnostics ELISA test kit, were used as the comparison samples between our one step ELISA and commercial ELISA test kit (Sanofi Pasteur). The results showed that 233 samples were positive by one-step ELISA and 245 samples were positive by commercial ELISA kit. The sensitivity specificity and accuracy of our test were 95.1%, 100% and 97.3%, respectively. While the positive and negative predictive values were 100% and 94.4%, respectively. Since the principle of our assay is based on monoclonal antibodies in a one-step assay, it gives advantages of time utilizing and simplicity over assays using heterologous antisera. This principle would also be applicable to a variety of antigen assay for which appropriate monoclonal antibodies are available.

Key words : hepatitis B virus, surface antigen, diagnosis, enzyme immunoassay

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Introduction

Hepatitis B virus infection causes significant morbidity and mortality in human. This hepatotrophic virus not only produces acute illness of varying clinical severity, but also can lead to chronic liver disease terminating in cirrhosis and death (Levine,1992).

Detection of serum hepatitis B surface antigen (HBsAg) has been shown to be of value in clinical practice, particularly for the diagnosis of hepatitis B viral infection (Skinhoj *et al.*,1997). This has been accomplished largely by several enzyme immunoassays employing the two-step sandwich method (Hollinger *et al.*,1971). However, the disadvantage of using the time-consuming two-step sandwich method have led to the development of one-step enzyme immunoassay as alternatives to two-step sandwich method (Liu and Green,1985; Kim *et al.*,1987). In the present study, we describe the production of a monoclonal anti-HBs and its application in one-step ELISA for the detection of serum HBsAg. The sensitivity and specificity of one-step ELISA was studied by comparing with the commercial ELISA test kit.

Materials and methods

Serum samples

Samples of human serum, from the National Blood Bank Center of Thai Red Cross Society, were previously screened for the presence of HBsAg using Abbott diagnostics HBsAg ELISA test kit. These specimens (245 HBsAg- positives and 202 HBsAg- negatives) were utilized as test samples.

Production of monoclonal antibodies

Female 8-week-old BALB/c mice were immunized intraperitoneally with 10 μg of Hepavax-B vaccine (Korea Green Cross Corporation, subtype adr) as the antigen. Mice were then boosted twice with the same dose of antigen after 3 and 5 weeks. Spleen cells of the immunized mice were taken 3 days after the last booster and then fused with P3-X63-Ag8.653 myeloma cells using polyethylene glycol 4,000. Clones secreting anti-HBs were identified by indirect ELISA and then subcloned by limiting dilution technique (Buddhirakkul *et al.*, 1998).

Quantities of the monoclonal antibody were produced by growing the hybridomas in ascites of pristane-primed BALB/c mice (Eryl and Cryer, 1991). Subsequently, the ascitic fluids were collected and antibodies were purified from the ascitic fluids by Affi-Gel protein A agarose (Bio-Rad).

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Coupling of monoclonal antibodies to a solid phase

Monoclonal antibodies were coated to 96-wells polystyrene plates (Nunc, Denmark) in pH 9.6 bicarbonate buffer overnight at 4°C. The plates were then washed several times with washing buffer and post-coated with blocking solution for 1 hour at 37°C. After being washed, the plates were dried by vacuum desiccator. These coated plates could be stored at 4° C for at least 4 months.

Enzyme-labelling of monoclonal antibodies

Monoclonal antibody protein concentration in milligrams per milliliter was determined from the absorbance at 280 nm. Purified monoclonal antibodies were conjugated to horseradish peroxidase (HRP, Sigma) by means of the modification of the glutaraldehyde method (Avrameas and Ternynck,1971). Briefly, purified monoclonal antibody was dialyzed against carbonate buffer at 4°C, overnight. Next, 10 mg of HRP was dissolved in 0.2 ml of 1.25% glutaraldehyde, followed by the addition of 0.5 ml of carbonate buffer. The solution was incubated overnight at room temperature. Then, HRP mixture was dialyzed overnight against carbonate buffer at 4°C. For conjugation steps, 5 mg of purified monoclonal antibodies in carbonate buffer was added into 0.7 ml of dialyzed HRP mixture and incubated overnight at room temperature. This mixture was then added with 0.1 ml of 0.2 M lysine and diluted into 1 : 2 with glycerol. The conjugate was divided into aliquots and stored at 4°C.

One-step assay for HBsAg

Fifty microliters of the serum control or serum samples with an equal volume of HRP-labelled monoclonal antibody were added into the coated plates and incubated at 37°C for 2 hours. The plates were washed five times with the washing buffer and 100 µl of tetramethylbenzidine substrate were added and incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 100 µl of 1% hydrochloric acid, and bound antibody activity was detected by measuring the optical density at a wavelength of 450 nm.

Commercial ELISA test kit

Commercially-available Sanofi Pasteur HBsAg ELISA kits were used in comparison with the one-step ELISA. The system of this kit was also a one-step sandwich ELISA using three monoclonal antibodies which adding the sample and the conjugate together. The system took about two hours and a half to get the result. The sensitivity and specificity of this commercial ELISA were 100% and 99.6%, respectively.

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Stability of one-step ELISA

For one-step ELISA, various reagents that may be critical to the stability were checked for the quality twice a month. Those are monoclonal antibodies coated microplates (strips) and HRP-conjugated monoclonal antibodies.

Results

The monoclonal antibodies specific to a common "a" determinant of HBsAg were obtained. Each of them was utilized as enzyme-labelled tracers, allowed a one-step ELISA to be developed and compared for sensitivity and specificity with a commercial HBsAg assay. It was found that monoclonal antibody purified from ascitic fluids with high titers of anti-HBs was suitable for the development of ELISA test for HBsAg. The conjugate from purified monoclonal antibody coupled with HRP by glutaraldehyde method could be obtained at a high titer (1:1,000), a small amount of monoclonal antibody (0.5 μg of monoclonal antibody / well) could be used to coat microplates. Moreover, the peroxidase-conjugated and the monoclonal antibody-coated plate were found to be stable at 4°C for at least 8 and 4 months, respectively.

When our one-step ELISA was compared with commercial ELISA test kit using 447 serum samples (245 positive, 202 negative), the result showed that 233 and 245 samples were positive by our one-step ELISA and the commercial ELISA kit, respectively. The same 233 samples were positive by both ELISA tests. (as shown in Table 1)

Table 1 Comparison of 447 samples tested for HBsAg by commercial ELISA and the one-step ELISA.

	one-step ELISA		
Commercial ELISA	**HBsAg positive**	**HBsAg negative**	**Total**
HBsAg positive	233 true positive(TP)	12 false negative (FN)	245
HBsAg negative	0 false positive (FP)	202 true negative(TN)	202
Total	**233**	**214**	**447**

The one-step ELISA technique for HBsAg was shown to have a sensitivity of 95.1% and specificity of 100% with a positive and negative predictive value of 100% and 94.4%, respectively. The accuracy of this one-step ELISA was 97.3%. (as shown in Table 2)

Table 2 Diagnostic values of the one-step ELISA test.

Sensitivity	TP / TP+FN = 95.1%
Specificity	TN / TN+FP = 100%
Accuracy	TP+TN / TP+TN+FP+FN = 97.3%
Positive predictive value	TP / TP+FP = 100%
Negative predictive value	TN / TN+FN = 94.4%

Discussion

Currently, there has been a great demand for sero-surveillance of infectious diseases in developing countries, especially for hepatitis. However, commercially-available ELISA test kit for HBsAg are too expensive for massive serum screening. Since the ELISA that had been proved to be one of the suitable technique for massive screening, then prompted us to develop our one-step ELISA test and had been used widely in our laboratory and the others provincial medical sciences centers.

In this study we introduced the method for the preparation of HRP monoclonal antibody conjugate using two-step glutaraldehyde method. This conjugation method led to an increase in assay sensitivity over assays using conjugates prepared by the one-step methods (Langone and Vunakis, 1981).

The selected substrate used in our ELISA was tetramethyl-benzidine (TMB) because its metabolites were non-mutagenic and non-carcinogen. The blue colour generated after reaction gave an excellent contrast to the background and accordingly gave a high sensitivity to the assays, especially those interpreted by eye. The endpoint determinations were often superior to those obtained by o-phenylenediamine dihydrochloride (OPD) substrate (Catty and Raykundalla,1989).

This study demonstrated that it is possible to develop a local-made ELISA test kit for HBsAg in Thailand; however further improvement to obtain better sensitivity of the assays for HBsAg. Future efforts will involve characterization of new hybrid cells and the development of the sensitivity of the one-step ELISA test.

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In conclusion, we have shown that the one-step ELISA test for HBsAg has special advantages. Because the whole system can be complete within two and a half hours. Another advantage of this technique is that the simplicity of the assay makes it suitable for automation which is an important feature when there are a lot of specimens to be tested. The preparation of the monoclonal antibody-coated plates is extremely simple and the coated plates can be stored for at least 4 months at $4^{\circ}C$.

Finally, the principle of a one-step, two-site assay using monoclonal antibodies used in this study is also applicable for the detection of other viral and protein antigens.

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