ABSTRACT

The aim of this study was to utilize the antibody produced using Indonesia local horses (Equus caballus) to make the conjugate of ELISA kit for detection of hepatitis B virus (HBV) surface antigen (HBsAg). The polyclonal antibodies were isolated and purified from local horses immunized repeatedly using isolated and purified HBsAg from Indonesia. The antibodies were conjugated with horseradish peroxidase by a modified method of Nakane and Kawai. The conjugate activities were performed using the principle of ELISA test conducted by the researchers as well as by independent laboratory. Commercial conjugate for HBsAg ELISA was used as a comparison study. The results of this study indicated that the antibody produced from local horses can be used to make conjugates that were comparable to commercial HBsAg ELISA kit.

Keywords: Local equine, ELISA, polyclonal antibody, hepatitis B, HBsAg

INTRODUCTION

Up to now the hepatitis B virus (HBV) remains a major cause of world health problems as a cause of significant morbidity and mortality (Zukerman and Zukerman, 2000). Around the world, over 350 million people are infected chronically with HBV, some of them will develop severe liver disease including cirrhosis and hepatocellular carcinoma (Mulyanto et al., 2011, 2012). The highest prevalence of HBV infection is generally found in Asia and Africa (Lee, 1997). In Indonesia the prevalence of HBV varies among the islands. It has been reported that HBV carrier rates among apparently healthy populations range from 4 to 20.3% (Khan et al., 2004; Mulyanto et al., 2008). Several efforts to prevent the spread of HBV have been conducted by the Indonesian government, such as through a national vaccination program of children and do the screening of blood donors carried out by the Indonesian Red Cross.

To perform the screening test, some standard test kits or methods such as enzyme-linked immunosorbent assay (ELISA) are required. Several standard test kits including ELISA are available in Indonesia. However, they mostly use components that are imported from overseas which may lead to elevated production cost. To anticipate the growing need, as well as to reduce our dependency on imported products, it is necessary to develop an ELISA kit against HBV
with raw materials produced locally.

Horse (*Equus caballus*) has long been used for immunological research (Markel, 2007; Simon, 2007). However, in Indonesia, the utilization of local horses for diagnostic purposes is still limited. In this paper, the results of the utilization of local horses as a source of polyclonal antibodies against HBV, which were then used as raw materials to develop an ELISA conjugate, are reported. It is expected that the conjugate can be used for the development of ELISA kits for screening tests of suspected samples being infected with HBV.

**MATERIALS AND METHODS**

**Chemicals and Equipment**

The enzyme horseradish peroxidase (HRP) Type VI-A (Lot No. 115 76701), ethylene glycol anhydrous 99.8% (Lot No. 0856AM), 1-fluoro-2,4-dinitrobenzene (FDNB; Lot No. 0001451175), sodium meta-periodate (NaIO4; Lot No. MKBCB727), 3,3’,55-Tetramethylbenzidine (TMB) Substrate (T4444 #089K1699), tween 20, BSA, were purchased from Sigma, USA, and sodium borohydride (NaBH4) obtained from Merck. Other chemicals such as NaCl, KH2PO4, were of analytical grade.

ELISA plates used were Microtiterwells, 12x8 (break apart) strips, 96 wells (SLP). Optical density measurements were made on a Thermo Scientific Multiscan EX Type 355 spectrophotometer (Shanghai, China).

**Polyclonal and Monoclonal Antibodies**

Anti HBV polyclonal antibody is produced locally (West Nusa Tenggara Hepatitis, WNTH Laboratory). It was derived from hyperimmune sera of adult local *Equus caballus* (Sumba and Lombok female horses) which multi-immunized with hepatitis B surface antigens (HBsAg) according to Mulyanto (1991). The polyclonal antibody was a gift from Prof. Mulyanto and used as the main material to develop the conjugate. The monoclonal antibody (WNTH Laboratory Lot No. KO436) which is anti “a” determinant specific to HBsAg was also a courtesy of Prof. Mulyanto. The antibody was used for coating the ELISA plates.

**ELISA Kit Commercial**

ELISA Kit commercial used as a ‘gold’ standard throughout this study was Wantai HBsAg ELISA Kit (Wantai, China).

**Conjugate Preparation**

Conjugate was prepared for three days continuously by coupling HRP-aldehyde solution with horse-anti HBsAg polyclonal antibody based on Nakane and Kawaoi (1974) with slight modifications. On day one, HRP-aldehyde solution was prepared by dissolving a 5 mg of HRP into 1.0 ml freshly made of 0.3 M sodium bicarbonate, pH 8.1. In to this solution, 0.1 ml of FDNB (1-fluoro-2,4-dinitrobenzene) in absolute ethanol was added, and then mixed gently for 1 hr at room temperature. Subsequently, 1.0 ml of 0.08 M NaIO4 was added and mixed gently for 30 min at room temperature. Following this, 1.0 ml of 0.16 M ethylene glycol was added, mixed gently for 1 hr at room temperature, and dialyzed against three 1-liter changes of 0.01 M sodium carbonate buffer, pH 9.5, at 4°C.

After the dialysis, a 5 mg of horse anti HBsAg-IgG was added to 3 mL of HRP-aldehyde solution and mixed gently for 3 hr at room temperature. After that, 5 mg of NaBH4 was added and let stand for 3 hr at room temperature, then dialyzed at 4°C against PBS overnight. Finally, the solution was purified by applying the solution to a 60 x 1.5 cm Sephacryl-200 HR column (Pharmacia, Sweden) equilibrated in PBS and the absorbance of the fractions was read at 280 nm. The main peak fractions was pooled and designated as HRP labeled horse-anti HBsAg IgG polyclonal antibody and used for conjugate of the in-house HBsAg ELISA.

**ELISA Plates Preparation**

ELISA plates were prepared by a standard procedure by coating the wells with 5µg/mL of mouse-anti HBsAg monoclonal antibody in carbonate buffer pH 9.5 at 100 µL per well and left overnight at 4°C. Afterward, plates were blocked with 1% BSA-PBS, 300 µL/well for 3 h at room temperature and washed 3 times with PBS containing 0.05% Tween 20 (PBS-T). Following washing, plates were dried and kept in a foil sachet with desiccate gel. The plates were stored in 4°C until used.

**Conjugate Tests**

The principle of conjugate test that had been developed from horse serum in this study was based on immunological reactions between the primary antibody coated to the plate and the antigen in the sample, and then the reaction to the
conjugate. To that end, 50 µL of three different (low, medium, and high) concentrations of in-house HBsAg positive or negative standards were added into each well of plates coated with the primary antibody. Furthermore into the respective wells were added 50 µL of conjugate. The plate was then sealed and incubated for 60 min at 37°C. After incubation, the plate was washed with a washing solution of 300 µL per well. Washing was performed 5 times, and 50 µL of TMB substrate was added into each well subsequently. Following incubation for 15 min in a dark room at 37°C, the reaction was stopped by adding 50 µL of 1 N sulfuric acid into the respective wells. The reaction was then read using an ELISA photoreader at a 450 nm wavelength.

Comparisons are performed simultaneously using a commercial ELISA Kit HBsAg. In addition, comparative test was also conducted by an independent laboratory with a commercial kit using a known concentration of HBsAg standard to obtain as objective result as possible. The HBsAg concentrations used were 0, 1.24, 5.43, 24.10, 107 and 465 IU/mL.

**Data Analysis**

The data obtained were compared descriptively to give an idea of the quality of conjugated antibody produced in this study. Quantitative data regarding the ELISA tests were tabulated and analyzed according to Drouet et al. (2003), and Depamede and Kisworo (2011).

**RESULTS AND DISCUSSION**

Based on historical records, the horse has long been used for research in the field of immunology. Even since scientists have not been able to discover about the antigen-antibody reactions, Emil von Behring had successfully used horse serum as a serum therapy for diphtheria (Stiehm and Johnston, 2005; Raju, 2006, Simon, 2007). Nowadays horses still been used for serum therapy purposes (Bunning et al., 2002; de Cuétos et al., 2011), and for diagnostic development such as diagnostic for HBsAg. In this study the purified polyclonal antibody produced in local horses using HBsAg of Indonesia origin (Mulyanto, 1991) was used to make conjugates for HBsAg ELISA. Preparation of the conjugates includes several steps such as binding of HRP enzyme as a marker onto the polyclonal antibodies. Following the binding, the conjugate was purified by means of size-exclusion column method. In the present study the binding and purification methods were performed by using the classical method according to Nakane and Kawaoi (1974) with some modifications, and a representative result is presented in Figure 1. The main peak (fractions from minute 55 to minute 75) is the image of the binding reaction between the antibody and the HRP enzyme. The pooled fractions was then defined as the in-house conjugate and used in subsequent experiments.

To find out whether the developed conjugates could be used as a reagent to detect HBsAg, the conjugates were tested against a negative and three defined in-house positive standard samples. The test results are shown in Figure 2. It can be seen in Figure 2 that the conjugates produced in this study gave comparable results to existing commercial kits (P >0.05). Furthermore, test results carried out by an anonymous independent laboratory (Figure 3) was also revealed that the conjugates produced in this study have a comparable quality to that of the commercial conjugate against known concentration of HBsAg standards used to perform the comparison tests. Pattern of curves presented in Figures 2 and 3 are in agreement with the results of Karakus et al. (2007).

According to Gerrits et al. (1991), key points to consider when developing a conjugate is process itself should not affect the activity of enzymes and antibodies used. The results of the present study indicated that the conjugate (pooled fractions of the main peak, Figure 1) provides immunological and enzymatic reactions that are
equivalent to a similar reaction on a commercial conjugate (Figure 2). Furthermore, the independent test results as shown in Figure 3 reinforced that the conjugates produced in this study gave reliable results. Even so, when it was seen in Figures 2 and 3 there was a tendency that the conjugates produced in this study (filled circles) was still below the commercial conjugate (open circles). Shin and Heo (2000) asserted that in addition to sensitivity and specificity of a diagnostic kit, the other values that need to also consider is detection limit and its reproducibility. For those reasons, the results of the present study still need to be followed up further.

Overall these results suggest that the antibodies produced by utilizing the local horses to develop the conjugate ELISA, can provide comparable results to conjugates that available commercially.

Utilization of Indonesia germ plasma is very important, especially in the efforts to develop vaccines and immunodiagnositics such as against HBV, especially to improve the immune specificity of the vaccine or the kit. Recently it has been reported that in Indonesia some new HBV genotypes have been elucidated (Lusida et al., 2008, Mulyanto et al., 2009, 2010, 2011, 2012). This means that efforts to prevent the spread of HBV in Indonesia will be more effective if we can provide the components to develop diagnostic kits derives from the local germ plasma. This should become a serious concern for Indonesia with a population of more than 200 million people with the HBV carrier rates are 4-20.3% (Khan et al., 2004; Mulyanto et al., 2008).

CONCLUSION

This study shows that antibody generated using the local horses can be utilized to develop test kit against infectious diseases such as Hepatitis B with feasible results. However, further studies are still required to improve it sensitivity, specificity, reproducibility and stability as needed by a diagnostic kit.

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