

Anti-HBsAg IgY polyclonal antibodies potential as capture antibody for HBsAg Detection Kit development

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ABSTRACT

Hepatitis B, affecting about 296 million globally, is a significant concern, with Indonesia ranking second in Southeast Asia for case numbers. The disease's latent initial phase, devoid of early symptoms upon hepatitis B virus (HBV) infection, highlights the demand for precise diagnostics. This research aimed to develop anti-HBsAg polyclonal antibodies (pAb) for application as capture agents within a sandwich enzyme-linked immunosorbent assay (ELISA). Chicken egg-derived IgY antibodies have advantages over mammalian ones due to simpler extraction and higher yield. In a study involving 21-week-old chickens, four intramuscular injections of 500 µg HBsAg antigen in Freund's adjuvant were administered at two-week intervals. Eggs as IgY source were collected daily and then IgY was isolated from egg yolk using polyethylene glycol (PEG) precipitation. The Bradford method was used to measure the total protein concentration, while the existence of IgY and pAb specific IgY against Anti-HBsAg was verified through SDS-PAGE and sandwich ELISA using HRP as a reporter, respectively. The resulting SDS-PAGE showed two distinct IgY bands: a 68 kDa heavy chain and a 23 kDa light chain. Using these anti-HBsAg IgY antibodies as capturing agents, the slightly elevation of IgY pAb against HBsAg level has been identified within the second week following the initial immunization. Subsequently, from the third to the eighth week, antibody levels escalated significantly, ranging from 2 to 13-fold higher than those observed in the second week. These findings suggest the potential use of IgY pAb as effective capture antibodies in sandwich ELISA for HBsAg antigen detection.

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1. Introduction

Hepatitis B is a disease caused by infection with the hepatitis B virus (HBV) and has a wide distribution (WHO, 2021). HBV infection can cause acute and chronic disease (liver cirrhosis and liver cancer). In 2019, 296 million people worldwide were living with chronic hepatitis B infection, with 1.5 million new infections every year (WHO, 2022). HBV has a high infection rate (highly contagious). There are several regions that have a fairly high rate of chronic HBV infection, one of which is the Southeast Asia Region (Lavanchy, 2004). According to the Ministry of Health of the Republic of Indonesia, Indonesia is the second country in Southeast Asia after Myanmar to have high cases of hepatitis B. It is estimated that around 24 million people are infected with hepatitis B and C viruses with 14 million of them suffering from chronic hepatitis B and C, and approximately 1.4 million people have the potential to get liver cancer (Kemenkes, 2013).

Hepatitis B is one of the dangerous diseases. In 2019, 820,000 cases of death in the world were caused by complications of hepatitis B, mainly due to cirrhosis and liver cancer (WHO, 2022). This number is higher than the number of deaths caused by HIV disease (WHO, 2021). This high mortality rate can be caused by the fact that most people infected with HBV do not experience any

symptoms (asymptomatic) early in the infection (Fong, et al., 1994). Symptoms of hepatitis B such as abdominal pain, jaundice, dizziness and vomiting appear when the patient has reached the stage of acute and even chronic infection. Therefore, it is necessary to have an early lab test to find out whether a person is infected with HBV or not. In laboratory tests, one of the biomarkers used for diagnosis is HBsAg antigen. This antigen appears at the beginning of infection, which is about 1-2 weeks after infection occurs, or at the latest at 11-12 weeks after infection and is produced in large quantities in serum in the early stages of infection (Liang, 2009). Early laboratory testing of HBsAg is necessary to prevent the dangerous complications caused by hepatitis B.

Based on these problems, this study was aimed to produce polyclonal Antibodies (pAb) which were expected to be used to detect HBsAg antigen, and to develop a sandwich enzyme-linked immunosorbent assay (ELISA). pAbs possess the ability to bind to multiple epitopes, making them highly suitable for various applications, including ELISA (Ascoli and Aggeler, 2018). When used as capture antibodies in sandwich ELISA, pAbs offer a wider sensitivity range compared to monoclonal antibody (mAb) mAb pairings, due to their enhanced capability in capturing multiple

antigen variants or epitopes presented by the analytes (Cox et al., 2012). This becomes especially crucial since antigens typically consist of multiple epitopes. Additionally, pAbs exhibit greater stability due to their biophysical diversity, which proves advantageous for ELISA diagnostic kit development when facing environmental challenges during transportation that may lead to inactivation, lability, or precipitation of other antibody forms (Ascoli and Aggeler, 2018). In this research, pAbs would be produced from chicken eggs. Chickens have immunoglobulin Y (IgY) which has a homologous function with IgG in mammals. IgY is present not only in the bloodstream but also within eggs, particularly in the yolk. The production of pAbs from chicken eggs has advantages when compared to the production of antibodies from mammalian serum because the antibody isolation process does not harm the animal, and easy to perform. On the other hand, the level of antibodies produced is higher than the level of antibodies produced from mammalian serum (Leenars et al., 1999).

In this study, HBsAg antigen was used for immunization of chickens. HBsAg antigen was injected intramuscularly in 21-week-old chickens four times (one priming immunization and three booster immunizations) with an interval of two weeks. Eggs were collected before and after immunizations. Eggs were stored at 4°C. IgY antibody was isolated from egg yolk by a PEG precipitation method and the presence of IgY was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The isolated IgY pAbs was used as capture antibody in the sandwich ELISA method. In the future, it is hoped that the sandwich ELISA method with IgY pAbs components could be used for early laboratory diagnostic test for hepatitis B disease.

2. Materials and methods

2.1. Materials

Three ISA Brown laying hens, aged 2-3 weeks, were acquired from Missouri Poultry shop in Bandung. These hens were raised and nourished with Phokphan feed. The vaccination process began when the hens reached 21 weeks of age, which was the point at which they commenced laying eggs in this research. HBsAg antigen was obtained from PT. Biofarma, was used for immunization and development of the sandwich ELISA. Complete Freund's Adjuvant (F5881) and Incomplete Freund's Adjuvant (F5506) from Sigma Aldrich were used as ingredients for immunizations. This research obtained ethical approval from the Health Research Ethics Commission, Padjadjaran University, No.894/UN6.C1.3.2/KEPK/PN/2016.

2.2. Immunization

An amount of 500 µg of HBsAg antigen dissolved in 500 µL of Freund's adjuvant was injected intramuscularly into 21-week-old chickens. Immunization was carried out four times with an interval between immunizations of two weeks. The immunization schedule is shown in Fig. 1. The adjuvant used in the first immunization was Complete Freund's Adjuvant (CFA), and the next 3 immunizations used Incomplete Freund's Adjuvant (IFA). Eggs were collected every day, starting from before immunization until the end of the study and stored at 4°C.

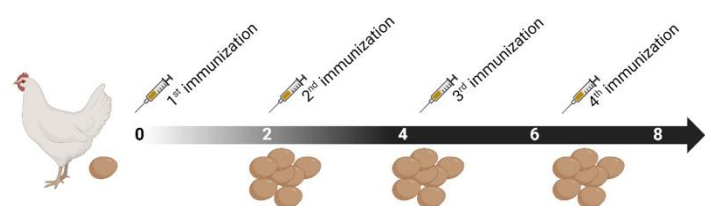


Fig.1. Chicken immunization schedule with HBsAg antigen. Chickens were immunized with 500 µg of HBsAg antigen dissolved in 500 µL of Freund's adjuvant three times. Eggs were collected every day.

2.3. IgY isolation from egg yolk

IgY protein was isolated by precipitation using polyethylene glycol (PEG) based on research conducted by Pauly et al. (2011). Immunized chicken eggs were collected and weighed daily. The egg yolk was separated from the egg white and was rinsed carefully with running water. The egg yolk was then placed on a paper towel to remove any remaining egg white. After that, the volume of the yolk was measured. The yolk was mixed with 3.5% (w/v) PEG 6000 (Sigma-Aldrich, US). The yolk solution was centrifuged for 15 minutes, 10,000 g at 4°C. The supernatant was filtered to obtain a clear filtrate. PEG 6000 8.5% (w/v) was added to the filtrate. The filtrate was centrifuged for 15 minutes, 10,000g at 4°C. The supernatant was discarded and the pellet was dissolved in phosphate buffer saline (PBS) to a final volume of 10 mL. PEG 6000 (12% w/v) was added to the solution and the yolk solution was centrifuged for 15 minutes, 10,000 g at 4°C. The supernatant obtained was discarded, then the pellet was dissolved in 2 mL PBS. The solution was centrifuged for 15 minutes, 10,000 g at 4°C. The supernatant which contained IgY were stored at -20°C. The protein concentration of the IgY solution was measured by the Bradford method (He, 2011).

2.4. Confirmation of IgY and IgY levels

The presence of IgY was confirmed by SDS-PAGE. After electrophoresis, the proteins were stained with Coomassie Blue. Furthermore, the presence of anti-HBsAg specific IgY pAb was confirmed by the sandwich ELISA method.

A total of 10 µg of IgY samples were coated onto wells of a 96-well plate, U-bottom with coating buffer/carbonate-bicarbonate buffer (pH 9.6), then incubated overnight at 4°C. The reagents in the wells were discarded, the remaining reagents in the wells were removed by rinsing two times using wash buffer (20x Glycine/Borate wash solution), with an incubation time of about one minute for each rinse, then 200 µL BSA-blocking buffer (pH 7.4) was added to the wells. The reagents were incubated for two hours at room temperature. The reagents were discarded, the remaining reagents were removed by rinsing twice using the same procedure, then 25 µL sample diluent (PBS) and 75 µL HBsAg antigen 1 µg/mL were added to the wells. The reagents were incubated for one hour at 37°C ± 1°C. The reagents were discarded, the remaining reagents were removed by rinsing three times using the same procedure, 50 µL of reagent containing mouse-IgG anti-HBsAg conjugated Horseradish-peroxidase (HRP) was added into the wells. The plate was shaken for ten seconds and incubated for 30 minutes at 37°C ± 1°C. The reagents were discarded, the remaining reagents were removed by rinsing five times using the same procedure and 100 µL substrate solution was added to the wells. The plate was incubated for 1-2 minutes and 50 µL of stop solution (1N H₂SO₄) was added. The absorbance of the solution was measured with a microplate reader at a wavelength of 450 nm.

2.5. Statistical analysis

The significance level of the IgY level data was analyzed using Tukey Post hoc in SPSS. The differences in anti-HBsAg IgY levels of each week were calculated at a significance level of p<0.05.

3. Results and discussion

3.1. Confirmation of the presence IgY

This study used IgY samples from egg yolks isolated by PEG precipitation based on Pauly et al. (2011). The level of isolated

protein obtained from the samples ranged from 8.48 to 20.36 mg/egg, with an average of 13.37 mg/egg. The presence of IgY in the samples was confirmed by SDS PAGE based on the presence of two main bands at 68 kDa and 23 kDa, which represented the Heavy Chain (HC) and Light Chain (LC) of IgY (Pauly et al., 2011; Han et al., 2012) (Fig. 2).

Isolated IgY was the total IgY contained in the egg yolk. The results of SDS PAGE of samples in the treatment group and the control group (Fig. 2) showed that the bands from week 0 to week 8 had almost the same thickness.

3.2. Level of anti-HBsAg IgY in IgY samples

ELISA was used to detect and measure the levels of specific IgY anti-HBsAg in the IgY samples every week until week 8. After 1st immunization, IgY anti-HBsAg could not be detected yet in the treatment group (Fig. 3). At week 2, which was about two weeks after the priming immunization in the immunization schedule, the

IgY anti-HBsAg could be detected. This was in accordance with research by Sudjarwo et al. (2012) that also showed that specific IgY was detected about two weeks after the first immunization. The absorbance of IgY anti-HBsAg at week 2 was 0.091 ± 0.057 . The level of anti-HBsAg IgY increased from week 2 to week 8 with a significant increase occurring at week 7 ($p < 0.05$). The increase in the level of anti-HBsAg IgY from the 3rd to the 8th week ranged from 2 to 13-fold compared to the level of anti-HBsAg IgY from 2nd week.

IgY anti-HBsAg is an adaptive immune response to the HBsAg antigen. Anti-HBsAg IgY was not detected one week after the priming immunization. This might be due to the length of time needed for the B cells to differentiate into IgY anti-HBsAg-producing plasma B cells in the germinal center (GC). In addition, anti-HBsAg IgY is transported from serum into the egg yolk during the development of oocytes in the ovaries. According to Patterson et al. (1962), specific IgY was detected in eggs four days after the appearance of specific IgY in serum.

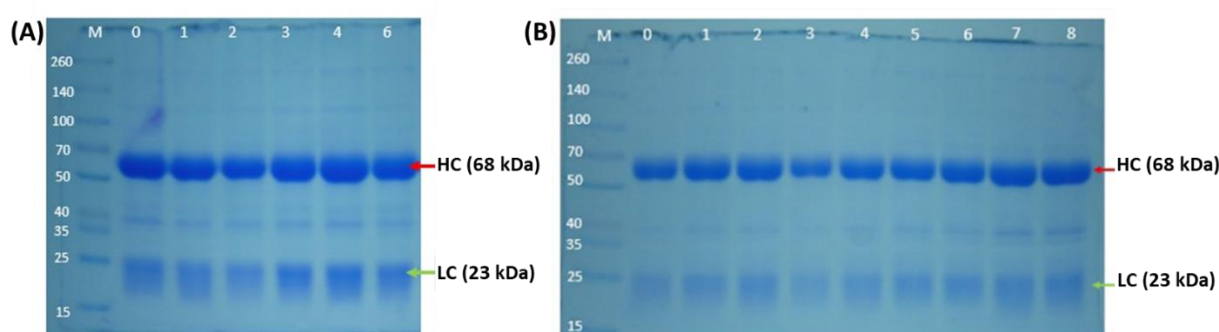


Fig. 2. SDS PAGE results of chicken IgY samples in the control (A) and treatment group (B) showed the presence of two main bands, namely the band for the Heavy chain (HC) and Light Chain (LC) which are the component of IgY. Lane M is a molecular weight marker; rows 0-6 or 8 show IgY samples from week 0 to week 6 (for control group) or 8 (for treatment group).

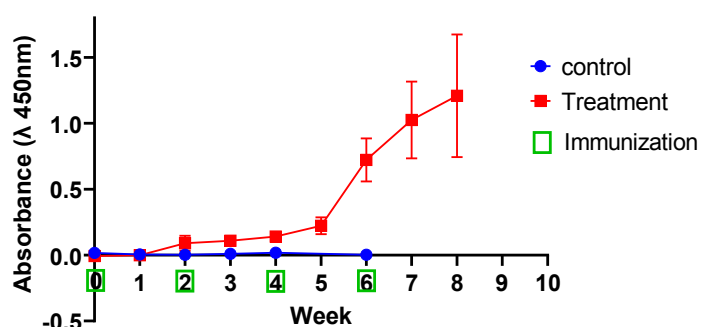


Fig. 3. Increase in the anti-HBsAg IgY levels in the treatment group from week 0 to week 8 and absence of anti-HBsAg IgY in the control group from week 0 to week 6. Anti-HBsAg IgY was measured using sandwich ELISA system at the wavelength of 450 nm.

Anti-HBsAg IgY was detected in the 2nd week. The increase in the level of anti-HBsAg IgY that occurred at week 3 to week 8 compared to week 2 indicated the influence of the second, third and fourth immunizations in inducing naive B cells to differentiate into plasma B cells that would produce anti-HBsAg IgY. In addition, there was also the influence of adjuvants that triggered an increase in the response of the adaptive immune system. According to Phan et al. (2006), B cells that differentiated and proliferated in the germinal center (GC), underwent gene conversion at the receptor site and class switching so that the IgY obtained high affinity for the HBsAg antigen.

3.3. Development of sandwich ELISA method for HBsAg antigen detection

In this study, the sandwich ELISA method was used to measure the level of anti-HBsAg IgY (Fig. 4). The results showed

that the anti-HBsAg IgY in the IgY samples could be coated onto wells in a 96-well plate (Fig. 5). The attachment of anti-HBsAg IgY to the well surface was caused by the passive adsorption of the IgY protein to the plastic surface, namely by hydrophobic interaction between the plastic surface and IgY (Crowther, 2009). The addition of coating buffer such as carbonate/bicarbonate buffer, pH 9.6 could optimize the bonding reaction that occurred. The 0th and 1st week samples and the negative control sample (K-) showed a pink color which indicated the absence of anti-HBsAg IgY in the IgY samples. The orange color in the samples from the 5th to the 8th week and a slight orange color at the 2nd to the 4th week indicated the presence of anti-HBsAg IgY in the IgY samples that were used to coat the wells as capture antibodies. This result indicated that IgY as capture antibody could recognize the antigen specifically.

Typically, mammals like horses, goats, rabbits, and rats have been used for the pAbs production. Nevertheless, this approach posed a drawback due to the potential suffering and mortality of these animals during the isolation of pAbs. Thus, exploring different animals like chickens offers a potential solution for pAb production. Chickens, for instance, generate IgY with analogous functions to mammalian IgG. Notably, this IgY is detectable not only in serum but also within chicken eggs. Previous studies had utilized chickens in the production of pAbs (Michael et al., 2010; Leenars et al., 1999; Schade et al., 2005). The use of IgY in antibody production had advantages, including the isolation method of IgY did not harm the animals because IgY was isolated from eggs instead of serum; maintenance and handling of chickens was easier and less expensive than mammals. Additionally, IgY titers in eggs surpassed those in serum, and chickens exhibited prolonged egg production (Muller et al., 2015). Moreover, the level of pAbs produced in chickens was higher than that of mammals. As much as 960 mg of IgG in 160 ml of rabbit blood

could be obtained over a four-month period and the amount of IgY that could be obtained from a chicken in the same period of time was up to two-fold higher (Santos et al., 2014). The amount of antibody that could be produced from a single chicken in one year was 40 g, with specific antibody levels produced around 1-10% (Sudjarwo et al., 2012; Santos et al., 2014). In this study, pAbs that specifically recognized HBsAg could be produced, which was shown by the increase in absorbance when compared to the control in the sandwich ELISA results. Using the polyclonal IgY antibodies as capture antibodies, it is possible to distinguish different concentrations of IgY antibodies against HBsAg after animals receive repeated vaccinations. This indicates the potential of polyclonal IgY antibodies against HBsAg to be used as capture antibodies in an ELISA system, but for further development, in the future it is necessary to determine the concentration of IgY used for a validated ELISA system.

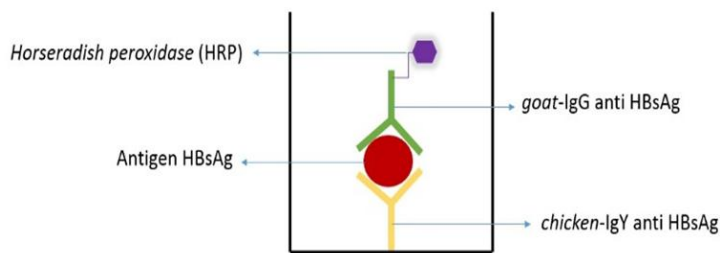


Fig. 4. ELISA sandwich arrangement for detection of isolated anti-HBsAg IgY. Chicken-IgY as capture antibody was coated in 96-well plates. HBsAg bound to Chicken-IgY against HBsAg specifically. HRP-conjugated-goat-IgG anti HBsAg was used as the secondary antibody.

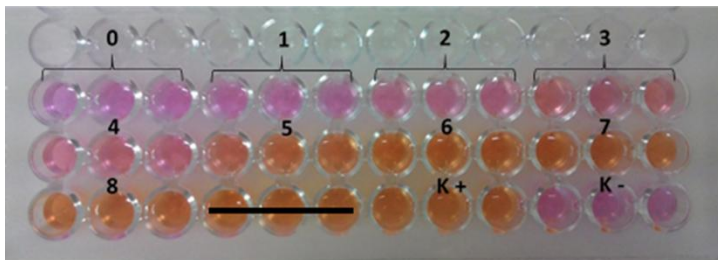


Fig. 5. ELISA results in the treatment group; code 0 to 8: IgY samples from egg isolated on week 0 to week 8 after immunization, K(+): positive control, K(-): negative control. Each group was analysed in triplicate.

4. Conclusion

From the results of this study, it could be concluded that the level of anti-HBsAg IgY increased significantly after four immunizations and the anti-HBsAg IgY could be used as a capture antibody to detect HBsAg antigen in the sandwich ELISA system. The best anti-HBsAg IgY antibodies that can be used for the developed sandwich ELISA are primarily two weeks after the third vaccination.

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Conflict of interest

The authors declare there is no conflict of interest in this study.

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