

ORIGINAL ARTICLE

PURIFICATION OF ANTI-HBsAg FROM EGG YOLKS OF IMMUNIZED HENS AND ITS APPLICATION FOR DETECTION OF HBsAg

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Background- The hen's egg yolk possesses an immunoglobulin known as immunoglobulin (Ig) Y, with an advantage over conventional antibody production of certain antigens. The aims of this study were to produce anti-hepatitis B surface antigen (HBsAg) in the egg yolk of hens immunized with hepatitis B vaccine and to coat anti-HBsAg to the wells of an enzyme-linked immunosorbent assay (ELISA) plate for detection of HBsAg in the sera of patients with hepatitis B virus infection.

Methods- A group of five hens were immunized intramuscularly with 10 µg of recombinant hepatitis B vaccine at zero, 21 and 80 days. Two weeks after the third dose of the vaccine, the egg yolks were collected. The anti-HBsAg (IgY) was purified using a two-step polyethylene glycol 6000 and one-step 4-M ammonium sulfate process, followed by column purification. The purified anti-HBsAg (5 µg/mL) was coated to the wells of ELISA plates for detection of HBsAg.

Results- Two weeks after the third dose of vaccine, all the hens' sera were positive for anti-HBsAg (IgY). The sensitivity and specificity were 73.1% and 85.7%, respectively.

Conclusion- HBV vaccine was immunogenic in hens and ELISA using the purified anti-HBsAg from the egg yolks was able to detect the HBsAg in the sera of patients infected with hepatitis B virus.

Keywords • antibody • chicken • egg yolk • hepatitis B • immunoglobulin

Introduction

The hen's egg yolk contains the immunoglobulin known as immunoglobulin (Ig) Y.¹ An ordinary egg yolk has been shown to contain an antibody level up to 25 mg/mL and is a good potential source of immunoglobulins.² The inoculation of hens with antigens (bacterial or viral) has been shown to generate high titers of specific antibodies in the egg yolk.³ Now the production of immunized eggs, has become an economical source of antibodies for various applications such as immunoassays, as well as for food ingredients.^{4,5} The significance of IgY is its application in the prevention of intestinal infections with microorganisms such as *Escherichia coli* and rotavirus among infants.^{6,7}

Hepatitis B virus (HBV) is a member of the Hepadna group.⁸ Infection with HBV may be transient and self-limited, or may be persistent in chronic carriers for life. Transient HBV infection is occasionally severe and may be fulminant. The sequelae of chronic infection, chronic active hepatitis, cirrhosis and hepatocellular carcinoma, account for the major proportion of the morbidity and mortality of hepatitis B infection.⁸ It is estimated that about 280 million people in the world are chronic HBV carriers and about 1 million will die annually due to HBV infection.⁸ The prevalence of HBV infection was reported to be 2.49% in Hamadan Province, Iran, between 1989 and 1991.⁹ So far, several types of specific antibodies have been developed in animals for use in the detection of HBsAg in humans.¹⁰ The chicken IgY does not interact with mammalian complement, Fc receptors or protein A.¹¹ Thus, the aim of this study was to produce anti-HBsAg in

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egg yolks and coat the purified anti-HBsAg onto wells of enzyme-linked immunosorbent assay (ELISA) plates for detection of HBsAg.

Materials and Methods

A group of five hens (32 weeks of age) were selected and kept isolated to produce eggs. The hens were injected intramuscularly with three doses of 10 µg of recombinant hepatitis B vaccine (Heberbiovac, Havana, Cuba) containing aluminum phosphate, on days 0, 21 and 80. All five hens tested positive for anti-HBsAg seroconversion 2 weeks after the third dose of the vaccine.

An anti-HBsAg test kit (Organon Teknika, Boxtel, Holland) was used for detection of anti-HBsAg among the immunized hens. The eggs were then collected and the yolks were separated from the whites. The purification of IgY was done as described by Polson.³ The egg yolks were treated with 3.5% polyethylene glycol (PEG) 6000 by adding it to the egg yolks and then incubating the mixture for 30 minutes with stirring. This was followed by centrifugation at 12,000 x g for 30 minutes. After centrifugation, the precipitate was discarded. The supernatant was treated with 12% PEG 6000 and stirred for 30 minutes, followed by 10 minutes of centrifugation at 12,000 x g. After centrifugation the supernatant was discarded and the precipitate was dissolved in phosphate-buffered saline (PBS), pH = 7.2, without NaCl overnight at room temperature.

Equal amounts of the dissolved precipitate and 4 M ammonium sulfate were mixed and stirred for 30 minutes, followed by 10 minutes of centrifugation at 12,000 x g. The supernatant was discarded and the precipitate was dissolved in PBS pH = 7.2 and then dialyzed against PBS pH = 7.2 without NaCl overnight. The final purification of anti-HBsAg (IgY) was done using Sephadex G-200 (Pharmacia, Uppsala, Sweden) columns. The purified anti-HBsAg fractions were collected. The purity of the anti-HBsAg was tested using sodium dodecylsulfate-polyacrylamide gel electrophoresis. Five µg/mL of purified anti-HBsAg (IgY) was coated onto the wells of ELISA plates (Nunc, Roskilde, Denmark) using carbonate bicarbonate buffer pH = 9.6, for detecting HBsAg. The results of this coated anti-HBsAg for detecting HBsAg were compared to the HBsAg test kit manufactured by Organon Teknika. The sensitivity and specificity of HBsAg detection using the purified IgY anti-HBsAg were calculated.

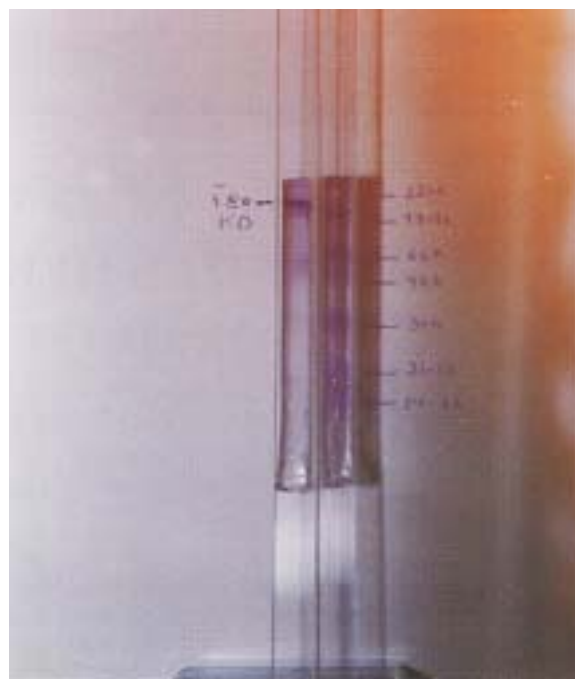


Figure. Purity of IgY anti-HBsAg from hen egg yolks by separation on sodium dodecylsulfate-polyacrylamide gel electrophoresis and detection by coomassie brilliant blue staining.

Results

All five immunized hens showed anti-HBsAg seroconversion after the three doses of HBV vaccine. The purified IgY from the egg yolks was positive for anti-HBsAg.

A highly purified IgY of 180 kDa was obtained (Figure). Five micrograms of anti-HBsAg coated to the wells of the ELISA plates showed very good sensitivity to absorbed maximum and minimum amounts of HBsAg in the sera of HBV infected patients.

There were 19 true positives, six true negatives, one false positive and seven false negatives in the detection of HBsAg using the purified hen IgY anti-HBsAg coated onto ELISA plates. The sensitivity and specificity for detection of HBsAg were 73.1% and 85.7%, respectively. The sensitivity and specificity of the HBsAg test kit manufactured by Organon Teknika for comparison were determined in our lab.

Discussion

In a recent study, the production and isolation of polyclonal immunoglobulins from egg yolks of hens immunized with Sendai virus were reported.¹²

A high titer of antibodies against influenza virus glycoproteins isolated from egg yolks of hens immunized with influenza A subunit vaccine was also reported,¹³ which implies the recent important application of egg yolk immunoglobulin.

Various techniques were used for the different purifications of IgY. The method described by Polson is a suitable technique for purification of IgY from egg yolks³ because lipoproteins and other fatty material in the egg yolk could be coagulated and removed by PEG 6000. It is also time consuming especially when dealing with large volumes of egg yolk.

Using complete and incomplete Freund's adjuvant with certain antigens stimulates high antibody titers in hen sera and egg yolks.^{6,7} In the present study, the recombinant hepatitis B vaccine with the adjuvant aluminum hydroxide (AL₂[OH]₃) also stimulated anti-HBsAg response in both sera and egg yolks of the immunized hens. Freund's adjuvant is most powerful in stimulating immune response and is used only for experimental animals, while the aluminum hydroxide adjuvant is a safe adjuvant used in human and animal immunization for stimulation of immune response.

Egg yolk IgY does not interact with mammalian complement, Fc receptors or protein A. Thus, it has a great advantage over polyclonal antibodies produced in mammals such as goats and rabbits.¹¹ Furthermore, antibodies isolated from yolk are stable during freezing and for at least three thawing/freezing cycles, with no loss of activity.¹⁴

In the studies by Polson, the Sephadex G-100 column was used for final purification.³ In the present study, the Sephadex G-200 column was used for the final purification of anti-HBsAg (IgY), while other techniques including Sepharose 4B, ion exchange, ultrafiltration and Sephadex G-100 could also be used.

The molecular weight of the purified anti-HBsAg was 180 kDa. Use of this antibody yielded a sensitivity of 73.1% and specificity of 85.7% in the ELISA for HBsAg. We suggest that immunization of purified HBsAg (contains s, pre-s1 and pre-s2)¹⁵ with complete and incomplete Freund's adjuvant in the hens is needed for future investigation.

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References

- 1 Akita EM, Nakai S. Immunoglobulin from egg yolk: isolation and purification. *J Food Sci.* 1992; **57**: 629-34.
- 2 Rose ME, Orlans E, Buttres SN. Immunoglobulin classes in the hen's egg: their segregation in yolk and white. *Eur J Immunol.* 1974; **4**: 521.
- 3 Polson A. Isolation of IgY from the yolks of eggs by a chloroform polyethylene glycol procedures. *Immunol Invest.* 1990; **19**: 253-8.
- 4 Song CS, Yu JK, Bai DH, et al. Antibodies to the alpha-subunit of insulin receptor of eggs of immunized hens. *J Immunol.* 1985; **135**: 3354-9.
- 5 Leslie GA, Clem LW. Phylogen of immunoglobulin structure and function. 3. Immunoglobulins of the chicken. *J Exp Med.* 1969; **130**: 1337-52.
- 6 Makoto S, Robert CF, Shuryo N. Anti-*E. coli* immunoglobulin in Y isolated from egg yolk of immunized chickens as a potential food ingredient. *J Food Sci.* 1998; **53**: 1361-5.
- 7 Yolken RH, Leister F, Wee SB, et al. Antibodies to rotaviruses in chickens eggs: a potential source of antiviral immunoglobulins suitable for human consumption. *Pediatrics.* 1988; **81**: 291-5.
- 8 Alfred MP. Hepatitis B virus: active and passive immunization. In: Stanley JC, ed. *Vaccines and Immunotherapy.* New York: Pergamon Press; 1991: 256-70.
- 9 Amini S, Mahmoodi MF, Andalibi S, Solati AA. Seroepidemiology of hepatitis B, delta and human immunodeficiency virus infections in Hamadan Province, Iran: a population-based study. *J Trop Med Hyg.* 1993; **96**: 277-87.
- 10 Malanchere-Bres E, Payatte PJ, Mancini M et al. CPG oligodeoxynucleotids with hepatitis B surface antigen (HBsAg) for vaccination in HBsAg transgenic mice. *J Virol.* 2001; **75**: 6482-91.
- 11 Lusch U, Schraner I, Wanke R, Jurgens L. The chicken egg, an antibody source. *J Vet Med.* 1986; **33**: 609-19.
- 12 Bizhanov G, Vyshniauskis G, Jonauskienė L. Production and isolation of polyclonal immunoglobulins from egg yolk of hens immunized with Sendai virus [in Russian]. *Balt J Lab Anim Sci.* 2000; **10**: 72-8.
- 13 Bogoyavlenskly AP, Berzin VE, Tolmachva VP, et al. Immunogenicity of influenza glycoproteins with different forms of supramolecular organization in hens [in Russian]. *Balt J Lab Anim Sci.* 1999; **4**: 99-105.
- 14 Hiek B, Hermann S. Rapid method of extraction of antibodies from hen egg yolk. *J Immun Meth.* 1984; **72**: 421-9.
- 15 Francis JM, Mark K. Hepatitis B vaccine. In: Plotkin SA, Orenstein WA, eds. *Vaccine.* 3rd ed. Philadelphia: WB Saunders; 1999: 158-82.