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Full Length Research Paper

Evaluation of the protective efficacy of immunoglobulin Y (IgY- antibodies) prepared against *Walterinnesia aegyptia* snake venom in Saudi Arabia

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Four groups of eight chickens were immunized intramuscularly with *Walterinnesia aegyptia* snake venoms mixed with Freund's complete adjuvant during the period from 1st October 2009 to 1st October 2011 at the Center of Excellence in Biotechnology Research, King Saud University, Saudi Arabia. Three weeks later, the injections were repeated with the venoms in incomplete Freund's adjuvant. Three boosters were given with the venoms at three weeks intervals. The immunoglobulin Y (IgY)-antibodies was extracted by ammonium sulphate-caprylic acid method, the antibody titer were tested by enzyme linked immunosorbant assay and the protective efficacies of the extracted immunoglobulins were performed. IgY-preparation extracted by ammonium sulphate-caprylic acid method showed lack of low molecular weight bands (non-immunoglobulin proteins) and the bands representing IgY-antibodies, which have molecular weights ranging from 180 to 200 kDa, appeared sharp and clear. Moreover, evaluation of the protective value of the IgY - antibodies prepared revealed that, one milliliter of extracted IgY-antibodies containing 15 mg/ml anti-*W. aegyptia* venom specific IgY could produce 100% protection against 50 LD_{50} and 75% protection against 60 LD_{50}. Laying hens could be used as an alternative source of polyclonal antibodies against *W. aegyptia* snake venoms due to several advantages as compared with mammals traditionally used for such purpose.

Key words: Snake venom, *Walterinnesia aegyptia*, immunoglobulins Y, protective efficacy, caprylic acid.

INTRODUCTION

Venomous snakebite is a worldwide problem in tropical regions and a serious medico-legal problem. Conventional antivenoms are prepared by immunizing large animals, usually horses, with individual venom or a range of different venoms obtained from several snakes to eliminate intraspecific variation (Theakston, 1996).

Chicken egg as an antibody source has attracted several investigators throughout the world for the non-invasive production of antibodies with applications in research, diagnosis and immunotherapy (Jensenius et al., 1981; Schade et al., 1991; Akita and Nakai, 1992; Larsson A and Sjoquist 1990; Zhang, 2003). The predominant class of immunoglobulin in chicken is called IgY, which is transferred from serum to the yolk for

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Abbreviations: SDS–PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis; **ELISA,** enzyme linked immunosorbent assay.

protection of the embryo against the infections (Larsson A and Sjoquist 1990). Due to the phylogenetic differences between avian and mammalian species, chicken antibodies also have biochemical advantages over mammalian ones. These differences increased sensitivity as well as decreased background in immunological assays. Additionally, chicken antibodies do not activate the human complement system, and does not react with rheumatoid factor, human anti-mouse IgG-antibodies or human Fc-receptor (Almeida et al., 2008). Moreover, maintenance and production of anti-snake venoms antibodies from horses is laborious and expensive. These advantages offered by avian egg yolk antibodies (IgY) over conventional mammalian antibody production are well documented (Jensenius et al., 1981; Schade et al., 1991; Akita and Nakai, 1992; Zhang, 2003). Therefore, due to their advantages, it was suggested that chicken antibodies would replace their mammalian counter parts in the future. The main goal of this study was to prepare and evaluate the protective efficacy of IgY prepared against *Walterinnesia aegyptia* snake venom located in the Saudi Arabian region.

MATERIALS AND METHODS

Immunization of chickens (laying hens) with snake venom

Chickens and animals

Five-month-old white leghorn female chickens (1.1 to 1.5 kg body mass), Swiss outbreed (18 to 20 g) mice, and rabbits (0.5 to 1.0 kg) were maintained in the animal facility of Experimental Animal Care and Research Center College of Pharmacy King Saud University, Saudi Arabia. Hens were used to produce IgY-antivenoms, Swiss outbreed mice were used to determine venom lethality, potency, the neutralizing potency of antivenom, in addition to other *in vivo* assay while rabbits were used to produce anti-IgY antiserum. Animal care was provided by expert personnel, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia). This method was according to Almeida et al. (2008).

Crude venoms from Saudi Arabian *W. aegyptia* snakes

The immunoglobulins-IgY were prepared against local Saudi Arabian *W. aegyptia*. The lethal dose – 50 LD_{50} of venom was estimated. The snakes supplied under the supervision of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

Determination of venom lethality

The lethal potencies of the venom (LD_{50}) were determined by i.p. injection of Swiss outbreed mice (18–20 g) using eight mice per group. Mortalities were recorded after 48 h, and LD_{50} was calculated. This method was according to Almeida et al. (2008).

Immunization schedule of chickens according to Almeida et al. (2008)

Groups of eight chickens were immunized intramuscularly in the breast region at two or three sites with 20 µg of *W. aegyptia* venoms, alone or mixed as indicated in Freund's complete adjuvant (FCA). Three weeks later, the injections were repeated with the venoms in incomplete Freund's adjuvant (IFA). Three boosters were given with the venom in 0.15 M NaCl by the same route, also at three-week intervals. Blood samples and eggs were collected before immunization to be used as negative controls either in immunochemical assays or in immunoprotection tests. Eggs were collected every day from each immunized chick and refrigerated at 4°C. Egg yolks were separated from the albumin and stored at −20°C.

Extraction and purification of IgY-antibodies from the egg yolk of immunized hens by different methods

The IgY- antibodies were extracted from the egg yolk of immunized hens by different methods to select the suitable method which yields highly purified immunoglobulins with effective neutralizing activity.

Extraction and partial purification using ammonium sulphate precipitation method

Yolks from 30 eggs of the same immunized group of hens were extracted by ammonium sulphate method explained by Almeida et al. (2008). Protein contents were adjusted to 10 mg/ml and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) (15%) and western blotted using rabbit serum anti-IgY as the first antibody. Anti-snake venom antibodies were quantified by the enzyme linked immunosorbent assay (ELISA) method, and their lethality neutralizing efficacies were assessed by *in vitro/in vivo* assays using Swiss outbreed mice as the animal test. IgY-immunoglobulins were similarly prepared and analyzed from pre-immunized egg yolks.

Extraction and purification using ammonium sulphate – caprylic acid method

The egg yolks were separated carefully from the egg white. One volume of whole egg yolk was dissolved in three volumes of phosphate buffered saline (PBS) pH 7.5 (1/4) dilution then the pH was adjusted to 4.6 with acetic acid and the non immunoglobulin proteins was precipitated by adding 6% caprylic acid (v/v). The precipitates obtained after centrifugation at 14000 × g for 30 min were discarded, the supernatants were collected and the pH was adjusted to 7.5 with 1 M Tris-buffer. Extractions of IgY-antibodies were carried out as previously mentioned by Almeida et al. (2008). The protein concentration was measured by Biuret method and the IgY-preparation was filtrated by 0.45 µm filter and stored at 4°C.

Characterization of egg antivenom IgY antibodies *in vivo* and *in vitro* and estimation of its neutralizing potency: SDS–PAGE and western blot analyses

Egg antivenom IgY antibodies were analyzed by western blot

Table 1. Comparison between the antibodies ELISA titers in serum samples and in IgY-antibody preparations from hens immunized with *W. aegyptia* at different time intervals post immunization.

Period (Week)	Immunization	Mean log_{10} antibody titer of the serum sample	Mean log_{10} antibody titer of the IgY- extracted by	
			Ammonium sulphate	Ammonium - caprylic acid
0	Pre-immunization	1.21 ± 0.16	1.09 ± 0.00	1.09 ± 0.00
2	2 weeks following primary immunization	2.18±0.16	1.73*** ± 0.02	1.87*** ± 0.23
4	2 weeks following 1st booster dose	2.84*** ± 0.13	2.27*** ± 0.17	3.0*** ± 0.17
6	2 weeks following 2nd booster dose	3.26*** ± 0.13	3.0*** ± 0.17	3.3*** ± 0.17
8	2 weeks following 3rd booster dose	3.62*** ± 0.16	3.7*** ± 0.17	4.0*** ± 0.17
10	2 weeks following 4th booster dose	3.32*** ± 0.16	3.4*** ± 0.17	3.9*** ± 0.17
12	2 weeks following 5th booster dose	3.02*** ± 0.16	3.2*** ± 0.00	3.6*** ± 0.17
14	-	2.66*** ± 0.13	3.0*** ± 0.17	3.3*** ± 0.17

**Moderately significant (p<0.01), ***highly significant (p<0.001), *non significant; SD, standard deviation, n = 3.

analysis and SDS-PAGE by the method explained by to Almeida et al. (2008).

Evaluation of antibody activity: ELISA method

Polystyrene ELISA plates (96 wells) were coated with 1.0 µg of native snake venom in 50 µl coating buffer (0.1 M carbonate bicarbonate, pH 9.6) and kept overnight at 4°C. The wells were washed once with PBS buffer containing 0.05% Tween-20. The wells were next blocked for 1.0 h at room temperature with 150 µl PBS buffer plus 1.0% gelatin. The wells were again washed three times with 300 µl washing buffer. Serial dilutions of IgY-preparations (1:1000 to 1:320,000) in PBS buffer plus 1.0% gelatin buffer containing 0.05% Tween-20 were prepared and 50 µl of each were added to individual wells and the plates were incubated at 37°C for 45 min. The wells were then washed five times with the same washing buffer. Rabbit peroxidase-conjugated anti-chicken IgY (whole molecule), diluted (1:800) in PBS buffer plus 1.0% gelatin buffer containing 0.05% Tween-20 (50 µl), were added to each well. The plates were incubated for 45 min at 37°C. After five washes with the washing buffer, 50 µl of substrate buffer (0.1 M citric acid, plus 0.2 M sodium diphosphate, 5.0 ml H_2O, 5.0 mg o-phenylenediamine dihydrochloride (OPD), 5 µl of H_2O_2) were added and incubated at room temperature for 10 to15 min. The reaction was terminated with 50 µl of 3 N sulfuric acid. Absorbencies were recorded at 492 nm using an ELISA plate reader. IgY from eggs collected before immunization were used as a negative control. Wells free of venom were used as blanks. The IgY dilution, giving an optical density of close to 0.2, was used to calculate the U-ELISA per milliliter of undiluted IgY solution. One U-ELISA is defined as the smallest amount of antibody giving an OD of 0.2 under conditions of ELISA assay. This method was carried out according to Pauly et al. (2009) and Zhen et al. (2008).

Determination of the neutralizing potency of IgY-antibodies prepared against snakes and scorpions venoms

The neutralizing potency of IgY-antibodies, produced along the immunization procedure, was evaluated according to the recommendation of WHO (1981) using groups of eight Swiss mice (18–20 g) for anti *W. aegyptia* venom antibodies.

RESULTS

Comparison between the anti *W. aegyptia* antibody ELISA titers in serum samples and IgY-antibody preparations from hens immunized with *W. aegyptia* venom at different time intervals post immunization

Two weeks after primary immunization with *W. aegyptia* significant increase (P< 0.001) in the mean $1og_{10}$, antibody titer was measured in tested serum samples against the venom toxin. Also a marked increase was recorded in the IgY-preparations, however, it was less significant (P<0.05). At six to eight weeks of immunization, the mean $1og_{10}$ antibody titer of the extracted IgY-antibodies reached to the relatively similar level of that of the serum samples, then it became higher and remained higher till the end of observation period as shown in Table 1 and Figure 1. At eight weeks post immunization (two weeks after the 3rd booster dose) the mean 1og antibody titer in tested serum samples was equal to 3.62 ± 0.16 as compared to a level of 3.7±0.17 and 4.0±0.17 in IgY preparations extracted by ammonium sulphate method or ammonium sulphate-caprylic acid method, respectively.

It was notable also that the antibody titers in the IgY-preparations extracted from ammonium sulphate-caprylic acid method were always higher than that recorded for IgY- preparations extracted from ammonium sulphate method as shown in Table 1 and Figure 1.

Protective value of *W. aegyptia* local Saudi snake venom specific IgY-antibodies as measured by neutralization test

Results demonstrated in Table 2 show that 15 mg/ml *W. aegyptia* specific IgY-antibodies produce 100% protection

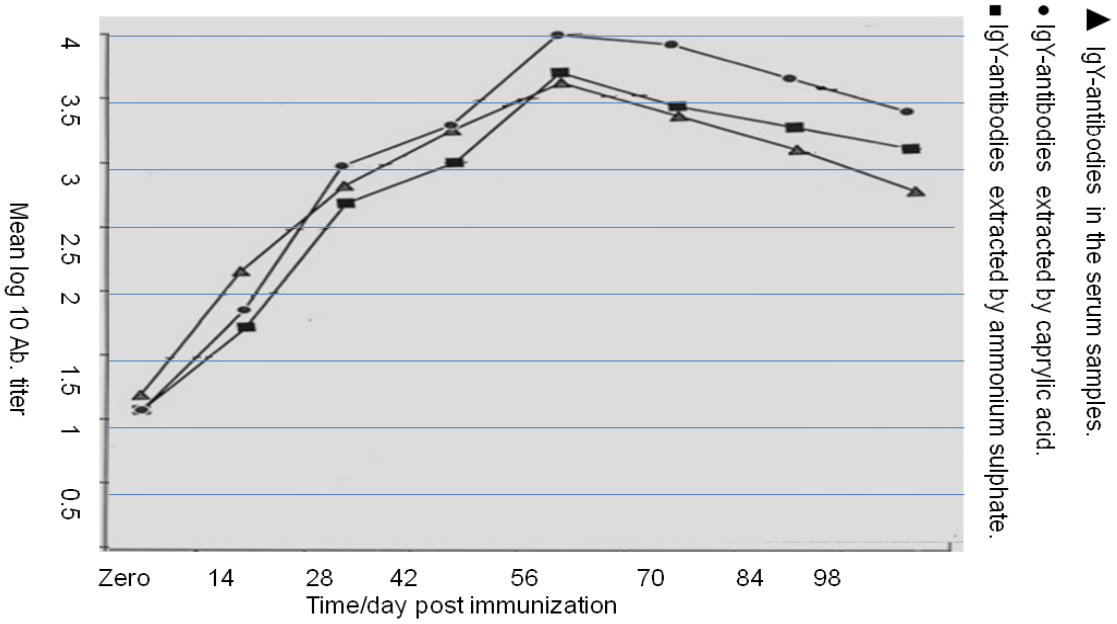

Figure 1. Comparison between the antibodies of ELISA titers in serum samples and in IgY antibody preparations from hens immunized with *W. aegyptia* at different time intervals post immunization.

Table 2. Neutralization test for measurement of the protective value of the IgY-antibodies prepared against *W. aegyptia* venom.

Venom potency (LD_{50})	Amount of venom in µg/0.5 ml saline (µl)	Amount of IgY used* (mg/ml)	No. of inoculated mice	Protection (%)
10	40	15	4	4/4 (100)
20	80	15	4	4/4 (100)
30	120	15	4	4/4 (100)
40	160	15	4	4/4 (100)
50	200	15	4	4/4 (100)
60	240	15	4	3/4 (75)
70	280	15	4	0/4 (0)

*****The venom and the specific IgY antibodies were mixed and incubated before injection of mice.

against 10, 20, 30 and 40 of *W. aegyptia* venom. This IgY concentration, however, produced 75% protection against 50 LD_{50} venom dose; with 60 LD_{50}, it gave 50% protection. No protection was induced with 70 LD_{50}. No mortalities were recorded among control mice that were injected with normal saline. Mice group inoculated with 2 LD_{50} of the venom showed 100% mortalities.

DISCUSSION

Chickens store high contents of IgY-antibodies in the yolk and are considered to be efficient antibody producers (Gottstein and Hemmeler, 1985; Almeida et al., 2008). The main goal of this project was to prepare and evaluate the protective efficacy of immunoglobulins (IgY) prepared against some snake venoms located in the Saudi Arabian region, in particular, local Saudi snake (*W. aegyptia*). The laying hens used in this study were divided into four groups immunized with the *W. aegyptia.* As compared with the levels of total protein content in serum samples collected from hens prior to immunization, significant increase ($P< 0.001$) was recorded in samples collected after two weeks of primary immunization. Boostering induced both increase and maintenance of higher levels of total protein in the examined serum samples from the immunized chicken groups. This increase continued up to the end of the observation period. The immunization-dependent increase in total protein content of serum can be attributed to the increased of production of

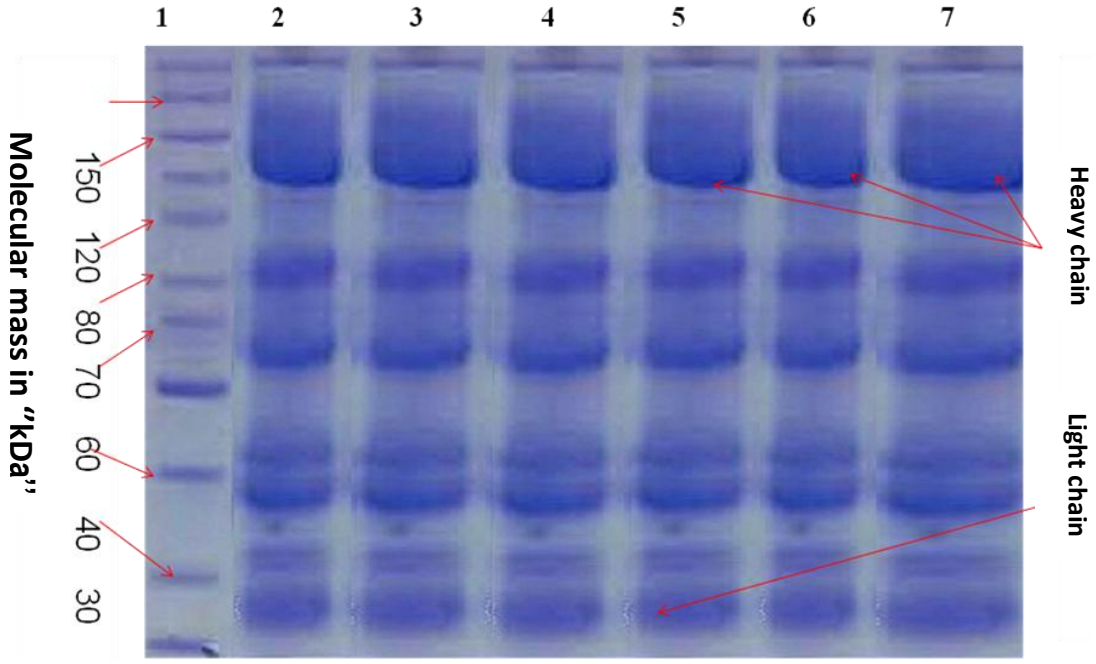

Figure 2. SDS-PAGE profile of crude immunglobulin-IgY prepared against *W. aegyptia* (lanes 2, 3 and 4) and *Echius carinatus* (lanes 5, 6 and 7).

immune-globulins and other immunoregulatory proteins by the immunocompetent cells. These results agree with those reported by Davalos-Pantoja et al. (2000) and Almeida et al. (1998).

The mean values of the total protein content of the IgY preparations extracted by ammonium sulphate method showed higher levels than those extracted by ammonium sulphate-caprylic acid method. This can be explained by the removal of non-immunoglobulin proteins from the IgY preparations through the effect of 6% caprylic acid (v/v) as shown in Figure 2. The effect of caprylic acid on purification and concentration of IgY has been declared through the SDS-PAGE analytical studies on IgY-preparations extracted by both methods. As compared with preparation extracted by ammonium sulphate method, IgY- preparation extracted by ammonium sulphate-caprylic acid method showed lack of low molecular weight bands (non-immunoglobulin proteins) and the bands representing IgY-antibodies, which have molecular weights ranged from 180 to 200 KD, appeared sharp and clear as shown in Figure 2. Similar results were reported by Polson et al. (1980), Akita and Nakai (1992), Mclaren et al. (1994) and Almeida et al. (1998).

Although, the total protein content of IgY preparations extracted by ammonium sulphate method was relatively higher than those extracted by ammonium sulphate-caprylic acid method, the titers of specific antibodies were significantly higher in IgY extracted by the later method. These results underline the value of incorporation of caprylic acid in production of purified IgY. The main objective of the present study was to evaluate the use of chickens as an alternative source for production of snake venom specific antiserum. It is worthy to realize that venom-specific antiserum, since it was first applied before 100 years, remains the only specific therapy for treatment of snake bites.

Venom specific antisera are mainly produced in horses, which is not only a costly process, but also is associated with animal suffering and severe side effects on the immunized horses. According to Thalley and Caroll (1990), a hen can produce 200 ml immunoglobulin/ kg/month, as compared with 10 ml/kg/month in case of a horse. All these points were behind the investigation done in the present study on the suitability of chickens as a cheaper, non-invasive source for production of polyclonal venom- specific antisera.

Analysis of results obtained with ELISA revealed that serum samples collected from hens immunized with *W. aegyptia* venom showed significant increase ($P< 0.001$) in the venom-specific antibodies after two weeks from the primary immunization. Through the effect of boostering; the anti-venom antibody levels reached a plateau at six to eight weeks from the primary immunization and remained significantly higher till the end of observation period. These results agree with those reported by Almeida et al. (1998), Sarker et al. (2001) and Almeida et al. (2008).

Evaluation of the protective value of the IgY - antibodies prepared against *W. aegyptia* venom revealed

that 1 ml of extracted IgY -antibodies containing 15 mg/ml anti *W. aegyptia* venom specific IgY could produce 100% protection against 50 LD_{so} and 75% protection against 60 LD_{50}. The neutralizing power of the produced anti venom IgY antibodies encourage the use of egg yolk as a cheap source of anti-venom polyclonal antibodies, particularly when the other numerous advantages of the IgY antibodies are considered. These results are similar to those reported by Carroll and Stollar (1983), Carroll et al. (1992), Almeida et al. (1998, 2008) and Sarker et al. (2001). From the reported literature and results recorded in the present study, laying hens are considered highly cost-effective source of polyclonal antigen specific antibodies as compared with mammals traditionally used for such production. Also due to the phylogenetic differences between avian and mammalian species, the use of IgY in immunological assays is associated with increase in the sensitivity and specificity.

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