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Preparation of the Immunoglobulin Y (IgY- antibodies) against Saudi *Echis carinatus* Snake Venom

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Saudi *Echis carinatus* bite is one of the leading causes of snake-bite mortality in Kingdom of Saudi Arabia. The traditional anti- *Echis carinatus* venom serum therapy prepared from mammales was found to be expensive and with high frequency of side effects. Therefore current study was aimed to generate a high titer immunoglobulin from egg yolk (IgY) of crude *Echis carinatus* venom immunized Leghorn hens, and to standardize an effective method for producing avian antivenom in relatively pure form. The IgY was isolated first by water dilution method to remove the lipid, then extracted by ammonium sulphate-caprylic acid method. The different purities of IgY from different isolating stages were submitted to enzyme-linked immunosorbent assay and SDS-PAGE. The LD50 of the *Echis carinatus* venom was 0.92 mg/kg body weight in mice. Twenty times the LD50 dose of venom was selected as challenge dose, and the ED50 of IgY was 15 mg IECF/mg venom. The results indicate that Laying hens could be used as an alternative source of polyclonal antibodies against *Echis carinatus* snake venoms due to several advantages as compared with mammals traditionally used for such purpose, thus possessing therapeutic significance for snakebite envenomation.

Key words: Snake venom, *Echis carinatus*, Immunoglobulins Y, Lethal dose 50, Caprylic acid.

Venomous snake bite 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 intra specific variation[1]

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. The predominant class of immunoglobulin in chicken is called IgY, which is transferred from serum to the yolk for protection of the embryo against the infections[2] . Because of 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, does not react with rheumatoid factor, human anti-mouse IgG antibodies or human Fc-receptor.[3]

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Polyvalent -Bitis and anti-Naja antivenom IgY antibodies produced by immunizing chickens with *B. arietans, B. nasicornis, B. rhinoceros, N. melanoleuca*, and *N. mossambica* venoms exhibited high antivenom activity (>100,000 U-ELISA/ml) as well as efficacy in neutralizing venom lethality (1,440 microgram of IgY neutralized 62.2 LD50 of venom), and were free of toxic products, pyrogens or bacterial and fungal contaminations[4].

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[3,5-8] .

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 is to prepare and evaluate the protective efficacy of immunoglobulins (IgY) prepared against *Echis carinatus* snake venom located in the Saudi Arabian region.

MATERIALS AND METHODS

Chickens and animals

Five-month-old white leghorn female chickens (1.1–1.5 kg body mass), Swiss outbreed (18–20 g) mice, and rabbits (0.5–1.0 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).

Crude venoms from Saudi Arabian *Echis carinatus* snakes

The immunoglobulins IgY were prepared against local Saudi Arabian snake venom; *Echis carinatus* snake venom. The lethal dose – 50 LD_{50} of venom will be 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 according to Almeida *et al*.[3]

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.

Immunization schedule of chickens according to Almeida *et al*.[3]

Groups of eight chickens were immunized intramuscularly in the breast region at two or three sites with 20 µg of *Echis carinatus* snake venom 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 3-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 will be 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.*, [3] Protein contents were adjusted to 10 mg/ml and analyzed by SDS–PAGE (15%) and Western blotted using rabbit serum anti-IgY as the first antibody. Anti-snake venom antibodies were quantified by the 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 yolk was separated carefully from the egg white. One volume of whole egg yolk was dissolved in three volumes of 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 14000xg for 30 minutes were discarded, the supernatants were collected and the pH was adjusted to 7.5 with 1M Tris-buffer.

Extraction of IgY antibodies were carried out as previously mentioned by Almeida *et al.*[3]The protein concentration was measured by Biuret method and the IgY-preparation was filtrated by 0.45µm filter and stored at 40C after being a liquoted in small test tubes.

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 analysis and polyacrylamide gel electrophoresis SDS-PAGE by the method explained with Almeida *et al.*[3]

Evaluation of antibody activity: ELISA method: (According to Pauly *et al.*, [9] and Zhen *et al.*, [10])

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 be washed three 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 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 OPD, 5µl of H_2O_2) were added and incubated at room temperature for 10–15 min. The reaction was terminated with 50 µl of 3 N sulfuric acid. Absorbance will be 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.

Determination of the neutralizing potency of IgY-antibodies prepared against *Echis carinatus* venoms

The neutralizing potency of IgY antivenom antibodies, produced along the immunization procedure, will be evaluated according to the recommendation of WHO (1981) using groups of eight Swiss mice (18–20 g) for anti *Echis carinatus* venom antibodies.

RESULTS AND DISCUSSION

The laying hens used in this study were divided into 4 groups immunized with the *Echis carinatus* snak venom.

As compared with the levels of antibody titer in the 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 antibody titer in the examined serum samples from the immunized chicken groups as shown in Table (1). This increase continued up to the end of the observation period which indicate that chickens store high contents of IgY-antibodies in the yolk and are considered to be efficient antibody producers.[3,11]

. The immunization-dependent increase in total protein content of serum can be attributed to the increased of production of immunoglobulins and other immunoregulatory proteins by the immunocompetent cells. These results agree with those reported by Davalos- Pantoja *et al.*[12] and Almeida *et al.*[13].

Antibodies specific to *Echis carinatus* snake venom started to appear in the tested serum

samples two weeks after primary immunization with a $1og_{10}$ antibody titer of 2.24±0.13. The ELISA antibody values reached a plateau (3.68± 0.16) at 2 weeks following 4th booster dose and remained significantly high (P< 0.001) up to the end of observation period (14 weeks). The measured antibody titers showed significant increase following the first, second, third booster doses. (P< 0.001), however, there was no difference between the third and the fourth booster dose as shown in Table (1). The obtained results indicate that chickens store high contents of IgY-antibodies

Table 1. Anti *Echis carinatus* venom antibody ELISA titers in serum samples collected from hens immunized with *Echis carinatus* venom at different time intervals post immunization

Period (Weeks)	Immunization	Mean Log_{10} Antibody titer X±SD*n*
Zero	Pre-immunization	0.00± 0.00
2	2 weeks following primary immunization	2.24±0.13
4	2 weeks following 1st booster dose	2.88***±0.16
6	2 weeks following 2nd booster dose	3.18***±0.16
8	2 weeks following 3rd booster dose	3.48***±0.16
10	2 weeks following 4th booster dose	3.68***±0.16
12	2 weeks following 5th booster dose	3.64***±0.13
14	-	2.84***±0.13

*** Highly significant (p<0.001). SD_n; stander deviation, n=5.

Table 2. Comparison between the anti Echis carinatus venom antibody ELISA titers in serum samples and in IgY-antibody preparations from hens immunized with Echis carinatus venom at different time intervals post immunization

Period (Weeks)	Immunization	Mean log10 antibody titer of the serum samples	Mean log10 antibody titer of the IgY extracted with	
			Ammonium sulphate	Ammonium - caprylic acid
Zero	Pre-immunization	0.00± 0.00	0.00± 0.00	0.00±0.00
2	2 weeks following primary immunization	2.24±0.13	1.88***±0.12	2.2***±0.17
4	2 weeks following 1st booster dose	2.88***±0.16	2.44***±0.17	2.68***±0.17
6	2 weeks following 2nd booster dose	3.18***±0.16	3.22***±0.17	2.84***±0.17
8	2 weeks following 3rd booster dose	3.48***±0.16	3.64***±0.17	4.0***±0.17
10	2 weeks following 4th booster dose	3.68***±0.16	3.82***±0.00	4.22***±0.00
12	2 weeks following 5th booster dose	3.64***±0.13	3.62***±0.17	3.8***±0.17
14	-	2.84***±0.13	3.42***±0.17	3.68***±0.17

** : Moderately significant (p<0.01). *** Highly significant (p<0.001).
*: Non significant. SDn; stander deviation, n=3.

in the yolk and are considered to be efficient antibody producers.[3,11]

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).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- 200 KD, appeared sharp and clear. Similar results were reported by Polson *et al*., [14]; Akita and Nakai [7]; Mclaren *et al*., [15] and Almeida *et al*., [13]).

As shown in Table (2), the mean $10g_{10}$ antibody titer started to increase in the serum samples and in the extracted IgY -antibodies after 2 weeks of immunization. The mean $1og_{10}$ antibody titer of the tested serum samples reached to 2.240.13after two weeks of primary immunization, as compared with 1.880.12 and 2.20.17in IgY - preparation extracted by ammonium sulphate method or ammonium sulphate-caprylic acid method, respectively.

The antibody titers in the extracted IgY-antibody preparations reached to the same level of that in the tested serum samples at 6 weeks of immunization, and then it increased and remained higher than the serum antibody titers till the end of observation period. The maximum antibody titers of the tested serum samples were observed at 8 weeks post immunization (2 weeks after 4th booster dose) and reached to 3.680.16mean $1og_{10}$ antibody titer. In the extracted IgY - antibody preparations.

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 [16] 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 *Echis carinatus* 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 6 - 8 weeks from the primary immunization and remained significantly higher till the end of observation period as shown in Table (2). These results agree with those reported by Almeida *et al*., [13]; Sarker *et al*., [17] and Almeida *et al*., [3]).

Evaluation of the protective value of the IgY - antibodies prepared against *Echis carinatus* venom revealed that one ml of extracted IgY - antibodies containing 15 mg/ml anti *Echis carinatus* venom specific IgY could produce 100% protection against 20 LD_{so} and 75% protection

Table 3. Neutralization test for measurement of the protective value of the IgY-antibodies prepared against *Echis carinatus* venom

Venom potencies	Amount of venom in µg/0.5ml saline	Amount of IgY used*	No. of inoculated mice	Protection %
10 LD_{50}	184 µl	15mg/ml	4	4/4 (100%)
20 LD_{50}	368 µl	15mg/ml	4	4/4 (100%)
30 LD_{50}	552 µl	15mg/ml	4	3/4 (75%)
40 LD_{50}	736 µl	15mg/ml	4	2/4 (50%)
50 LD_{50}	920 µl	15mg/ml	4	0/4 (0%)

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

against 30 LD_{50} as shown in Table (3). This IgY concentration, however, produce 50% protection against 30 LD_{50} venom dose; with 40 LD_{50} it gave 50% protection. No protection was induced with 50 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 [18]; Carroll *et al*., [19]; Almeida *et al*[13].,; Sarker *et al*., [17] and Almeida *et al*., [3]). 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.

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 increased the sensitivity and specificity.

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