Protective efficacy of immunoglobulins Y prepared against *Cerastes cerastes* snake venom in the Kingdom of Saudi Arabia

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ABSTRACT

الأهداف: تحضير وتقيم القوه الوقائية للأجسام المناعية (IgY) ضد سم الحية المقرنة الموجودة بالمملكة العربية السعودية.

الطريقة: أُجريت هذه الدراسة خلال الفترة من أكتوبر 2009م إلى أكتوبر2011م بمركز التمييز البحثي في التقنية الحيوية، جامعة الملك سعود، الرياض، المملكة العربية السعودية حيث تم تحصين 4 مجموعات من 8 دجاجات في العضل باستخدام السم المجمع من الحية المقرنة الموجودة بالمملكة العربية السعودية وذلك بعد خلطه مع Freund's complete adjuvant. وبعد 3 أسابيع تم تكرار التحصين ولكن باستخدام السم المخلوط بالمادة incomplete Freund's adjuvant، وتلاها التحصين بثلاثة جرعات منشطة من السم بمفرده على فترات ثلاث أسابيع بين كل جرعة منشطة. وقد تم استخلاص الأجسام المناعية Y من مح البيض المجمع من الدجاج المحصن باستخدام طريقه أمونيوم حمض الكبريتات الكابريلك. وتم قياس نسبة الأجسام المضادة باستخدام اختبار الإليزا وقياس القوه المناعية للأجسام المضادة المستخلصة في حيوانات التجارب.

النتائج: أشارت نتائج الدراسة إلى أن الأجسام المناعية المحضرة بطريقه كبريتات الأمونيوم مع حمض الكابريلك هي أفضل من تلك المحضرة بطريقه كبريتات الأمونيوم فقط مع عدم وجود بروتينات منخفضة الوزن الجزيئي (بروتينات غير مناعية). كما ظهرت البروتينات المعبرة عن الأجسام المناعيةY والتي يتراوح الوزن الجزيئي لها 200–180 كيلو دالتون أكثر وضوحاً. علاوة على ذلك أسفرت القوة الوقائية للأجسام المضادة Y أن واحد مللي من مستخلص IgY يحتوي على 15 ملغ/مل من الأجسام المضادة لسم الحية المقرنة ويمكن أن تنتج حماية بنسبة100% مقابل 50 LD50.

خاتمة: أظهرت هذه الدراسة بأن الدجاج الذي ينتج البيض يعد من المصادر العالية المردود من الأجسام المضادة البولي كلونل للعديد من المستضدات بالمقارنة مع الثدييات التي تستخدم عادة لإنتاج مثل هذه الأجسام المضادة.

Objectives: To prepare and evaluate the protective efficacy of immunoglobulin Y (IgY) prepared against local Saudi *Cerastes cerastes* snake venom.

Methods: The study was conducted between October 2009 and October 2011 at the Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Kingdom of Saudi Arabia. The study designed as follow; 4 groups of 8 chickens were immunized intramuscularly with *Cerastes cerastes* snake venoms mixed with Freund's complete adjuvant. Three weeks later, the injections were repeated with the venoms with incomplete Freund's adjuvant. Three boosters were given with the venoms at 3 weeks intervals. The IgY 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.

Results: Immunoglobulin Y preparation extracted by ammonium sulphate-caprylic acid method showed lack of low molecular weight bands. The bands representing IgY-antibodies, which have molecular weights ranged from 180-200 KD, appeared sharp and clear. Furthermore, evaluation of the prepared protective value of IgY-antibodies revealed one ml of extracted IgY-antibodies containing 15 mg/ml anti *Cerastes cerastes;* specific IgY could produce 100% protection against 50 LD50.

Conclusion: Laying hens could be used as an alternative source of polyclonal antibodies against *Cerastes cerastes* snake venoms due to several advantages as compared with mammals.

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The uses of chickens as an alternative source for polyclonal antibody production against a variety of antigens has been described.[1,2] Chickens are highly cost-effective producers of antibodies as compared to mammals, which are traditionally used for the same purpose.[3] The predominant class of immunoglobulin in chicken is called immunoglobulins Y (IgY), which is transferred from serum to the yolk for protection of the embryo against infections.[4] Because of the phylogenetic differences between avian and mammalian species, chicken antibodies also have biochemical advantages over mammalian. These differences include increased sensitivity as well as decreased background in immunological assays. Additionally, chicken antibodies do not activate the human complement system; do not react with rheumatoid factor, human anti-mouse IgG antibodies or human Fc-receptors.[1] Moreover, maintenance and production of snake venoms antibodies from horses is laborious and expensive. Polyvalent-Bitis and anti-Naja antivenom IgY antibodies produced by immunizing chickens with *Bitis arietans*, *Bitis nasicornis*, *Bitis rhinoceros, Naja melanoleuca*, and *Naja 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.[1] Antibody against *Common cobra* (*Naja naja*) and *Krait venoms* (*Bungarus caeruleus*) were effective in neutralizing the toxic and enzymatic activities of the venoms. The LD50 of venom in 18 g of mice was found to be 10 microgram for Cobra and 3 microgram for Krait venoms. The median effective dose (ED50) of anti-Cobra venom was 4.48 mg/5LD(50) and 1.0 ml neutralized 0.127 mg of Cobra venom and the median effective dose (ED50) of anti-Krait venom was 3.18 mg/5LD50) and 1.0 ml neutralized 0.051 mg of Krait venom. These results indicate that antivenom generated in chicken could be used for therapeutic purposes in case of snake bite envenomation.[2] Therefore, due to their advantages, it was suggested that chicken antibodies could replace their mammalian counter parts in the future. The purpose of this study was to prepare and evaluate the protective efficacy of immunoglobulin Y (IgY) prepared against local Saudi *Cerastes cerastes* snake venom.

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Methods. The study was conducted between October 2009 and October 2011 at the Center of Excellence in Biotechnology Research, King Saud University, Riyadh, Kingdom of Saudi Arabia. Firstly, immunization of chickens (laying hens) with *Cerastes cerastes* snake venom was performed according to Almeida et al.[1] 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 the Experimental Animal Care and Research Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Hens were used to produce IgY-antivenoms, Swiss outbreed mice were used to determine venom lethality, the neutralizing potency of antivenoms, in addition to other *in vivo* assay while rabbits were used to produce anti-IgY antiserum. Animal care was provided by an expert personnel, in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research[1] and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Saudi Arabia).

The IgY were prepared against local Saudi *Cerastes cerastes* snake venom. The lethal dose-50 (LD50) of the venom was determined by intraperitoneal injection of Swiss outbreed mice (18-20 g) using 8 mice per group following the methods recommended by the World Health Organization (WHO).[5] The snakes were supplied under the supervision of the Experimental Animal Care Center (College of Pharmacy, King Saud University, Riyadh, Kingdom of Saudi Arabia).

Vaccination schedule of chickens was performed according to Almeida et al.[1] Four groups of 8 chickens were immunized intramuscularly in the breast region at 2 or 3 sites with 20 µg of snake venoms mixed in Freund's complete adjuvant (FCA). Three weeks later, the injections were repeated with the venoms in incomplete IFA. Three boosters were given with the venoms in 0.15 M NaCl by the same route, also at 3 weeks 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 as described by Almeida et al.[1]

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 was performed by collecting the yolks from 30 eggs collected from the same immunized group of hens followed by extraction of IgY-immunoglobulins by ammonium sulphate method.[1] Protein contents were adjusted to 10 mg/ml and analyzed by sodium dodecyl sulfate 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 ELISA method, and their lethality neutralizing efficacies were assessed by *in vitro*/*in vivo* assays using Swiss outbreed mice as the animal test. Immunoglobulins-Y were similarly prepared and analyzed from pre-immunized egg yolks as described by Paul et al.[6]

Extraction and purification using ammonium sulphate-caprylic acid method: egg yolks were separated carefully from the egg white. One volume of whole egg yolk was dissolved in 3 volumes of phosphate buffer saline (PBS) pH 7.5 (1/4) dilution then the pH was adjusted to 4.6 with acetic acid, and the non-immunoglobulin proteins were 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. The protein concentration was measured by Biuret method and the IgY-preparation was filtrated by 0.45µm filter and stored at 4^0C after being liquoted in a small test tubes. The SDS-PAGE and Western blot analyses; egg antivenom IgY antibodies were analyzed by Western blot analysis and SDS-PAGE by the method explained with Almeida et al.[1] Evaluations of antibody activity occur using ELISA according to Pauly et al[7] and Zheng.[8] 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 will be washed once with PBS buffer containing 0.05% Tween-20. The wells were next blocked for 1.0 hour at room temperature with 150 µl PBS buffer plus 1.0% gelatin. The wells were again being washed 3 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 will be added to individual wells and the plates were incubated at 37°C for 45 min. The wells were washed 5 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 5 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 was recorded at 492 nm using an ELISA plate reader. The IgY from eggs collected before immunizations were used as a negative control. Wells free of venom were used as blanks. The IgY dilution, giving an optical density of close to 0.21, was used to calculate the unit of ELISA per milliliter of undiluted IgY solution. One unit of ELISA is defined as the smallest amount of antibody giving an optical density of 0.2 under conditions of ELISA assay.

The neutralizing potency of IgY antivenom antibodies, produced along the immunization procedure, were evaluated according to the recommendation of WHO[5] using groups of 8 Swiss mice (18-20 g) for each antivenom antibodies. Dried standardized venom solution in sterial normal saline (1mg/ml) was prepared. Different potencies from the LD50 were prepared as shown in **Table 1**. The IgY antibodies preparations (15mg total protein/ml) were added to each of the prepared mixtures (prepared from each venom potency) were incubated for 30 minutes in water bath at 37°C, then each mixture was injected intravenously into a group of 4 mice (0.5 ml/mice). The animals were kept under observation for 24 hours. The potency of IgY preparations was equal to the number of LD50 used

Table 1 - Neutralization test for measurement of the protective value of the immunoglobulin Y (IgY)-antibodies prepared against *Cerastes cerastes* venom.

Venom potencies	Amount of venom in µg/0.5ml saline	Amount of IgY used*	No. of inoculated mice	Protection (%)
10 LD_{50}	83 µl	15mg/ml	4	4/4 (100)
20 LD_{50}	166 µl	15mg/ml	4	4/4 (100)
30 LD_{50}	249 µl	15mg/ml	4	4/4 (100)
40 LD_{50}	332 µl	15mg/ml	4	4/4 (100)
50 LD_{50}	415 µl	15mg/ml	4	3/4 (75)
60 LD_{50}	498 µl	15mg/ml	4	2/4 (50)
70 LD_{50}	581 µl	15mg/ml	4	0/4 (0)

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

when 80% protection was achieved according to the recommendation of WHO.[5]

Statistical analysis was performed with the SPSS Statistical Package version 12.0 (SPSS Inc, Chicago, IL). *P*-values were calculated using the Mann-Whitney U and a *p*-value of <0.05 was considered statistically significant.

Results. The results of ELISA on serum samples and IgY-antibody preparations of hens immunized with *Cerastes cerastes* venom at different time intervals post immunization. As shown in **Table 2** and **Figure 1**, 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 2.18±0.16 after 2 weeks of primary immunization, as compared with 1.78±0.23 and 2.1±0.17 in 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 until the end of the observation period. The maximum antibody titers of the tested serum samples were observed at 8 weeks post immunization (2 weeks after the third booster dose) and reached to 3.68±0.16 mean $1og_{10}$ antibody titer. In the extracted IgY-antibody preparations, it was recorded after 10 weeks of immunization (2 weeks after the fourth booster dose) and reached to 3.8±0.00 and 4.1±0.00 in IgY-antibody preparations extracted by ammonium sulphate method and ammonium sulphate-caprylic acid method, respectively. The amount of anti-*Cerastes cerastes* IgY produced per hen is 200 mg immunoglobulin /kg/ month, and if its calculated per day it is 6.7mg/kg body weight. The SDS-PAGE analysis of the IgY preparations extracted by ammonium sulphate-caprylic acid method showed lack of low molecular weight bands (non-immunoglobulin proteins) if it is compared with the IgY preparation extracted by ammonium method only and the bands representing IgY-antibodies, which have molecular weights ranged from 180-200 KD, appeared sharp and clear as shown in **Figure 2**.

The protective value of *Cerastes cerastes* venom specific IgY-antibodies as measured by neutralization test. Results demonstrated in **Table 2** showed that 15mg/ml

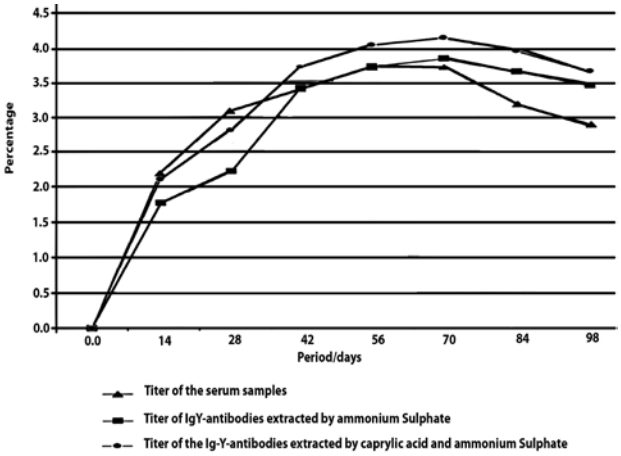

Figure 1 - Comparison between the anti *Cerastes cerastes* venom antibody titers in serum samples and in IgY-antibody preparations from hens immunized with *Cerastes cerastes* venom at different time intervals post immunization.

Table 2 - Comparison between the anti *Cerastes cerastes* venom antibody enzyme-linked immunosorbent assay (ELISA), titers in serum samples and in immunoglobulin Y (IgY)-antibody preparations from hens immunized with *Cerastes cerastes* venom at different time intervals post immunization.

Period (weeks)	Immunization	Mean log_{10} antibody titer of the serum samples	Mean log_{10} 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	2.18±0.16	1.78±0.02	2.1±0.17
4	2 weeks following 1st booster dose	3.08±0.062	2.2±0.17	2.8±0.17
6	2 weeks following 2nd booster dose	3.38±0.16	3.4±0.17	2.7±0.17
8	2 weeks following 3rd booster dose	3.68±0.16	3.7±0.17	4.0±0.17
10	2 weeks following 4th booster dose	3.68±0.16	3.8±0.00	4.1±0.00
12	2 weeks following 5th booster dose	3.14±0.13	3.6±0.17	3.9±0.17
14	-	2.84±0.13	3.4±0.17	3.6±0.17

P-values were calculated using the Mann-Whitney U. A $p<0.05$ was considered statistically significant.
*non-significant, †Moderately significant ($p<0.01$), ‡highly significant ($p<0.001$).

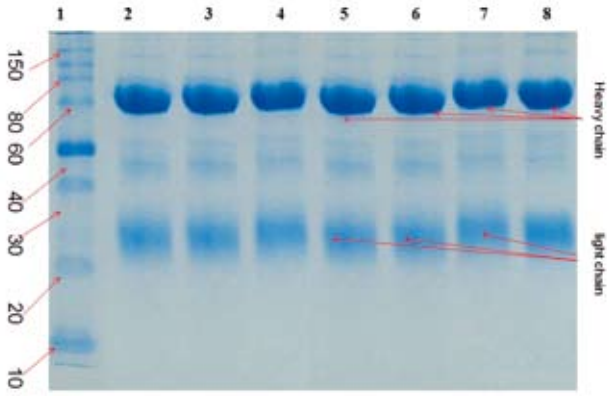

Figure 2 - Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel electrophoresis (profile of immunoglobulin-IgY prepared against *Cerastes cerastes* lanes extracted by ammonium sulphate-caprylic acid method.

Cerastes cerastes specific IgY-antibodies produced 100% protection against 10, 20, 30, 40 and 50 LD50 of *Cerastes cerastes* venom. This IgY concentration, however, produced 75% protection against 60 LD50 venom dose; with 70 LD50, no protection was induced. No mortalities were recorded among control mice that were injected with normal saline. Mice group inoculated with 2 LD50 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.[1,10] The laying hens used in this study were immunized with *Cerastes cerastes* snake venom. As compared with the levels of total protein content in serum samples collected from hens prior to immunization, a significant increase (p<0.001) was recorded in samples collected after 2 weeks of primary immunization. Boostering induced both increase and maintenance of higher levels of total protein in the examined serum samples from the 3 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 immunoglobulins and other immuno-regulatory proteins by the immunocompetent cells. These results agree with those reported by Davalos- Pantoja et al[11] and Almeida et al.[12] 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 ranging from 180-200 kD, appeared sharp and clear as shown in **Figure 2**. Similar results were reported by Polson et al,[13] Akita and Nakai,[14] Mclaren et al,[15] and Almeida et al.[12]

Although the total protein content of IgY preparations extracted by the ammonium sulphate method was relatively higher than those extracted by the 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 the production of purified IgY. Actually, incorporation of caprylic in the extraction of IgY antibodies could help in the removal of low molecular weight proteins (non specific immunoglobulin's), still there are some residue of low molecular weight proteins in the extracted IgY preparations.

It is worthy to realize that venom-specific antiserum, since it was first applied 100 years ago, 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 mg immunoglobulin /kg/month, as compared with 10 mg/ kg/month in case of a horse. All these points were behind the investigation carried out 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 *Cerastes cerastes* venom showed significant increase (p<0.001) in the venom-specific antibodies after 2 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 until the end of observation period. These results agree with those reported by Almeida et al,[1] Sarker et al,[17] and Almeida et al.[1] Evaluation of the protective value of the IgY-antibodies prepared against *Cerastes cerastes* venom revealed that one ml of extracted IgY-antibodies containing 15 mg/ml anti *Cerastes cerastes*

venom specific IgY could produce 100% protection against 50 LDso and 75% protection against 60 LDso. These results are similar to those reported by Almeida et al,[12] Sarker et al,[17] and Almeida et al.[1] From the obtained results it is recommended for future studies to prepare the IgY-antibodies against other Saudi snakes and scorpion venoms and to use the ammonium sulphate-caprylic acid method for extraction of immunoglobulin's.

Incorporation of caprylic in the extraction of IgY antibodies could help in the removal of low molecular weight proteins (non specific immunoglobulin's), still there are some residue of low molecular weight proteins in the extracted IgY preparations.

In conclusion, the laying hens are considered highly cost-effective source of polyclonal antigen specific antibodies as compared with mammals traditionally used for such production. One ml of extracted IgY-antibodies containing 15 mg/ml anti *Cerastes cerastes* venom specific IgY could produce 100% protection against 50 LDso.

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