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Immunological Properties of Anti *Naja haje arabica* (The Arabian Cobra) Snake Venom Antibodies Prepared in Chicken

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ABSTRACT
Naja haje arabica (Arabian cobra) is the major cause of snake-bite mortality in Kingdom of Saudi Arabia. The treatments of the snake bite envenomation occur by anti-snake venom produced in horses previously immunized with a mixture of venom. Therefore, one of the main objectives of the current study is to produce anti *Naja haje arabica* immunoglobulin in high titer from the yolk of chickens by immunization of two groups of white leghorn chickens (24 weeks old) with 30 μg of *Naja haje arabica* emulsified in Freund's Complete Adjuvant. Chickens has been immunized with booster doses of increasing concentrations of venom at two weeks' time intervals to increase the antivenom titer in the egg yolk. The characteristic IgY band of 180 kDa was observed on SDS-PAGE of the final extracted product. The ELISA antibody values reached the plateau at 2nd weeks following 4th booster dose and remained significantly high up to the end of observation period. The measured antibody titers showed significant increase following the first, second, third booster doses. However, there were no differences between the third and the fourth booster doses. Western blot technique was used to evaluate the specificity of antivenom IgY antibodies. The LD_{50} of the *Naja haje arabica* venom has been found to be 0.4 mg kg^{-1} body weight of white Swiss mice and 100% protection against 40 LD_{50} of *Naja haje arabica* venom could be obtained by15 mg mL^{-1} anti *Naja haje Arabica* specific IgY. The neutralizing power of the anti *Naja haje arabica* venom IgY and the absence of pyrogen, bacterial and fungal contaminations or toxic products, encourage the use of egg yolk as a cheap source of anti-venom polyclonal antibodies.

Key words: *Naja haje*, antivenom, Western blot technique, Immunoglobulins Y, lethal dose 50

INTRODUCTION

Venomous snakebite is a one of the most important problems in tropical countries and a serious medico-legal problem. Many venomous snakes of medical importance inhabit the Kingdom of Saudi Arabia and surrounding areas, where several of the most lethal snakes are found (Gasperetti, 1976, 1988). *Naja haje arabica* (the Arabian cobra) and *Walterinnesia aegyptia* (the Egyptian black cobra; the desert cobra) are representatives of the Elapidae family.

Naja is a genus of venomous elapid snakes known as cobras. Several other genera include species commonly called cobras (for example the King cobra, *Ophiophagus hannah* and the rinkhals or ring-necked spitting cobra,

Hemachatus haemachatus) but of all the snakes known by that name, members of the genus *Naja* are the most widespread and the most widely recognized as cobras. Various species occur in regions throughout Africa, Southwest Asia, South Asia and Southeast Asia.

Until recently the genus *Naja* had 20-22 species, but it has undergone several taxonomic revisions in recent years. 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 (Theakston, 1996; Larsson and Sjoquist, 1990). The maintenance and the production of anti snake venoms antibodies from horses are laborious and expensive. The advantages offered by avian egg yolk antibodies (IgY) over the conventional mammalian antibody production are well documented (Jensenius *et al*., 1981; Akita and Nakai, 1993; Schade *et al*., 2001; Zhang *et al*., 2004; De Almeida *et al*., 2008).

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 μg of IgY neutralized 62.2 LD_{50} of venom) and were free of toxic products, pyrogens or bacterial and fungal contaminations (Meenatchisundaram *et al*., 2008).

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 *Naja haje arabica* snake venom located in the Saudi Arabian region.

MATERIALS AND METHODS

Chickens and animals: During the period of 10th February to 30th May, 2014, 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 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. Rabbits were used to produce anti-IgY antiserum.

Crude venoms from Saudi Arabian *Naja haje arabica* snakes: The immunoglobulins IgY were prepared against local Saudi Arabian snake venom, *Naja haje arabica* snake venom. The Lethal Dose-50 (LD_{50}) of venom will be estimated.

Evaluation of the lethal dose 50 of the venom: The lethal dose 50 of the *Naja haje arabica* (LD_{50}) were evaluated by intra-muscular injection of five groups of Swiss outbreeds mice (20-22 g) eight mice per each group using. After 48 h the mortality rates and the LD_{50} were calculated according to De Almeida *et al*. (2008).

Immunization schedule of chickens: Two groups of eight chickens were immunized intra-muscularly in the breast region at two or three sites with 30 μg of *Naja haje arabica* snake venom venoms, alone or mixed as indicated in Freund's Complete Adjuvant (FCA) (De Almeida *et al*., 2008). Blood samples and eggs were collected before immunization to be used as negative controls either in immunochemical assays or in immunoprotection tests. The eggs of the immunized hens were collected every day and kept in refrigerators at 4°C. The egg yolks were separated from the albumin and kept at -20°C.

Extraction and purification using ammonium sulphate-caprylic acid method: The yolk from the immunized hens was separated from the egg white carefully and the IgY antibodies were extracted as previously described by De Almeida *et al*. (2008). The total protein content was determined by Biuret test and the IgY-preparations were filtrated using 0.45 μm filter and stored at 40ºC 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 by De Almeida *et al*. (2008).

Evaluation of antibody activity: The antibody titer was estimated by indirect ELISA using polystyrene ELISA plates after coating with 0.5 μg of native *Naja haje arabica* venom after mixing with 50 μL carbonate bicarbonate coating buffer and kept in the refrigerator at 4°C. Antibody titer was estimated according to according to Pauly *et al.* (2009) and Zhen *et al.* (2008).

Evaluation of the neutralizing potency of *anti Naja haje arabica* venoms

IgY-antibodies: The neutralizing potency of IgY antivenom antibodies, produced along the immunization procedure, will be evaluated according to the recommendation of Gottstein and Hemmeler (1985) using groups of eight Swiss mice (18-20 g) for anti *Naja haje arabica* venom antibodies.

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

RESULTS

Serum samples from hens immunized with *Naja haje arabica* venom were assayed with an ELISA just before immunization and every two weeks of immunization and boostering up to 14 weeks. Antibodies specific to *Naja haje*

Table 1: Comparison between ELISA titers of *Naja haje arabica*-specific antibodies in serum samples and Ig Y-antibody preparations from hens immunized with *Naja haje arabica* vaccines at different time intervals post immunization

Period (weeks)	Mean log_{10} antibody titer X±SD_n	Mean log_{10} antibody titer of the IgY-antibody extracted by ammonium-caprylic acid
0	1.27±0.160	1.09±0.00
2	1.52±0.112	1.19±0.17*
4	2.96±0.134***	2.60±0.00**
6	3.62±0.164***	3.60±0.17**
8	3.62±0.164***	3.80±0.00***
10	3.02±0.134***	3.40±0.17***
12	2.66±0.581***	3.10±0.17***
14	2.56±0.164***	3.20±0.17±***

**Moderately significant (p<0.01), ***Highly significant (p<0.001), *Non significant. SD_n; Standard deviation, n = 3

arabica appear in the serum samples of the immunized hens after two weeks from the primary immunization. Significant increase in the mean log_{10} antibody titer of tested serum samples was recorded 2 weeks post immunization and reached to 1.52± 0.112, as compared to a pre-immunization level of 1.27±0.016 (p<0.01). With respect to IgY-preparations, a significant increase in the mean $1og_{10}$ antibody titer of the *Naja haje arabica* specific IgY-antibody was first observed after 4 weeks of immunization (2 week after the 15 t b'20ster dose) and reached 2.6±0.00 in IgY preparations pre-immunization level of 1.09±0.00. At 8-10 weeks of immunization, the *Naja haje arabica*-specific antibody titers measured in the IgY- extracts were relatively similar to that in the serum, then its level exceeds that of the serum samples and till the end of the immunization period.

The maximum antibody titers of the tested serum samples were observed at 8 weeks post immunization (2 weeks after 4th booster dose) as shown in Table 1.

The protective value of *Naja haje arabica* local Saudi snake venom specific IgY-antibodies as measured by neutralization test revealed that 15 mg mL^{-1} *Naja haje arabica* specific IgY-antibodies produce 100% protection against 40 LD_{50} of *Naja haje arabica* venom.

DISCUSSION

Snakebite is one of the most important problems in tropical countries, therefore, the current study is aimed to evaluate the immunological properties of immunoglobulins- IgY antibodies prepared by immunization against local Saudi *Naja haje arabica* snake venom.

The 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 till the end of the immunization period. 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 (Davalos-Pantoja *et al*., 2000; Almeida *et al*., 1998). Similar results were reported by Polson *et al.* (1980), Almeida *et al.* (1998), Akita and Nakai (1993) and Mclaren *et al.* (1994).

Analysis of results obtained with ELISA revealed that serum samples collected from hens immunized with *Naja haje arabica* venom showed significant increase in the specific antibodies against the snake venom after two weeks from immunization (p<0.001). The anti-venom antibody reached the maximum level after 6-8 weeks by the effect of boostering and remain significantly higher till the end of immunization period (Almeida *et al*., 1998; Akita and Nakai, 1993; De Almeida *et al*., 2008; Polson *et al*., 1980; Mclaren *et al*., 1994; Sarker *et al*., 2001).

Evaluation of the protective efficacy of anti *Naja haje arabica* venom antibodies revealed that 1 mL of IgY-antibodies (15 mg mL^{-1} anti *Naja haje arabica* IgY) could protect 40 LD_{50} (100% protection and 75% protection against 50 LD_{50}). The neutralizing power produced by anti *Naja haje arabica* IgY antibodies encourage the use of such immunoglobulin as a cheaper source of anti *Naja haje arabica* IgY antibodies, similar to those reported by many authors (Carroll and Stollar, 1983; Almeida *et al*., 1998; Carroll *et al*., 1992; Sarker *et al*., 2001; De Almeida *et al*., 2008).

CONCLUSION

Naja haje arabica (the Arabian cobra) are the most dangerous and the most widely recognized local Saudi cobras. Therefore, this study was aiming to produce IgY-antibodies prepared against such venom. The protective value of *Naja haje arabica* local Saudi snake venom specific IgY-antibodies by neutralization test revealed that 15 mg mL^{-1} *Naja haje arabica* specific IgY-antibodies produce 100% protection against 40 LD_{50} of *Naja haje arabica* venom. So, this study will be helpful in further immunological investigations to produce specific IgY antibodies against more other Saudi snake venoms.

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REFERENCES

Akita, E.M. and S. Nakai, 1993. Comparison of four purification methods for the production of immunoglobulins from eggs laid by hens immunized with an enterotoxigenic *E. coli* strain. J. Immunol. Methods, 160: 207-214.

Almeida, C.M.C., M.M. Kanashiro, F.B.R. Filho, M.F. Mata, T.L. Kipnis and W.D. da Silva, 1998. Development of snake antivenom antibodies in chickens and their purification from yolk. Vet. Rec., 143: 579-584.

Carroll, S.B. and B.D. Stollar, 1983. Antibodies to calf thymus RNA polymerase II from egg yolks of immunized hens. J. Biol. Chem., 258: 24-26.

Carroll, S.B., B.S. Thalley, R.D.G. Theakston and G. Laing, 1992. Comparison of the purity and efficacy of affinity purified avian antivenoms with commercial equine crotalid antivenoms. Toxicon, 30: 1017-1025.

Davalos-Pantoja, L., J.L. Ortega-Vinuesa, D. Bastos-Gonzalez and R. Hidalgo-Alvarez, 2000. A comparative study between the adsorption of IgY and IgG on latex particles. J. Biomater. Sci. Polym., 11: 657-673.

De Almeida, C.M.C., C.L. da Silva, H.P. Couto, R.D.C.M. Escocard and D.G. da Rocha *et at*., 2008. Development of process to produce polyvalent IgY antibodies anti-African snake venom. Toxicon, 52: 293-301.

Gasperetti, J., 1976. *Walterinnesia aegyptia* Lataste-a rare black snake. J. Saudi Arab. Nat. Hist. Soc., 1: 2-8.

Gasperetti, J., 1988. Snakes of Arabia. Fauna Saudi Arabia, 9: 169-450.

Gottstein, B. and E. Hemmeler, 1985. Egg yolk immunoglobulin Y as an alternative antibody in the serology of echinococcosis. Zeitschrift Parasitenkunde, 71: 273-276.

Jensenius, J.C., I. Andersen, J. Hau, M. Crone and C. Koch, 1981. Eggs: Conveniently packaged antibodies. Methods for purification of yolk IgG. J. Immunol. Methods, 46: 63-68.

Larsson, A. and J. Sjoquist, 1990. Chicken IgY: Utilizing the evolutionary difference. Comp. Immunol. Microbiol. Infect. Dis., 13: 199-201.

McLaren, R.D., C.G. Prosser, R.C.J. Grieve and M. Borissenko, 1994. The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine α-lactalbumin. J. Immunol. Methods, 177: 175-184.

Meenatchisundaram, S., G. Parameswari, A. Michael and S. Ramalingam, 2008. Neutralization of the pharmacological effects of Cobra and Krait venoms by chicken egg yolk antibodies. Toxicon, 52: 221-227.

Pauly, D., M. Dorner, X. Zhang, A. Hlinak, B. Dorner and R. Schade, 2009. Monitoring of laying capacity, immunoglobulin Y concentration and antibody titer development in chickens immunized with ricin and botulinum toxins over a two-year period. Poult. Sci., 88: 281-290.

Polson, A., M.B. von Wechmar and M.H. van Regenmortel, 1980. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol. Commun., 9: 475-493.

Sarker, S.A., T.H. Casswall, L.R. Juneja, E. Hoq, I. Hossain, G.J. Fuchs and L. Hammarstrom, 2001. Randomized, placebo-controlled, clinical trial of hyperimmunized chicken egg yolk immunoglobulin in children with rotavirus diarrhea. J. Pediatr. Gastroenterol. Nutr., 32: 19-25.

Schade, R., I. Behn, M. Erhard, A. Hlinak and C. Staak, 2001. Egg Yolk Antibodies, Production and Application: IgY-Technology. Springer Lab Manual, Berlin, ISBN-13: 9783540666790, Pages: 255.

Theakston, R.D.G., 1996. Clinical Features of Envenoming from Snakebites. In: Envenomings and their Treatments, Bon, C. and M. Goyffon (Eds.). Fondation Marcel Merrieux, New York, pp: 117-126.

Zhang, J., J. Tanha, T. Hirama, N.H. Khieu and R. To *et al*., 2004. Pentamerization of single-domain antibodies from phage libraries: A novel strategy for the rapid generation of high-avidity antibody reagents. J. Mol. Biol., 335: 49-56.

Zhen, Y.H., L.J. Jin, J. Guo, X.Y. Li, Y.N. Lu, J. Chen and Y.P. Xu, 2008. Characterization of specific egg yolk immunoglobulin (IgY) against mastitis-causing *Escherichia coli*. Vet. Microbiol., 130: 126-133.