Extraction and characterization of polyclonal egg yolk antibodies (IgY) in chicken against cobra (*Naja naja*) venom

R. Rajendra Prabhu[1,4,*], Nalini Ramamurty[2] and A. Mathiyalagan[3]

[1]Department of Antitoxin, King Institute of Preventive Medicine, Chennai 600 032, India
[2]IVD, World Health Organisation, South East Asia Region, New Delhi 110 002, India
[3]Mediclone Research Centre, Chennai 600 048, India
[4]Present address: Department of Toxinology, Mediclone Research Centre, Chennai 600 048, India

Use of polyvalent antisera of equine origin has been existing as a proven treatment against snake envenomation. The need for alternative sources for antivenom production prompted this study. Polyclonal antibodies (IgY) extracted from egg yolk after inoculation of chickens with cobra venom revealed that IgY is functionally equivalent to IgG of equines in both yield and potency of venom neutralization.

Keywords: Cobra venom, IgY, neutralization, polyclonal antibodies.

IN India about 100,000 people are bitten by poisonous snakes and approximately 10,000 people die annually due to envenomation[1]. Hospital-based figures are unlikely to be accurate as most victims seek traditional treatment and deaths at home may go unrecorded. In India, among 236 species of snakes, 50 species are reported to be venomous. The most notable venomous species include cobra (*Naja naja*), common krait (*Bangarus caerulus*), Russell's viper (*Vipera russelli*) and saw scaled viper (*Echis carinatus*).

To date, the only medically proven treatment against snake poison is the equine polyvalent antiserum. The production of antisera (polyclonal antibodies) involves the immunization of the horse by injecting snake poison and subsequently collecting blood from the horse. There is always a constant demand for evaluating newer methods of production of safe antivenom and a need for a more refined technology[2].

Towards this end, avian egg yolk has been identified as a cheap, abundant and noninvasive source of immunoglobulins. Collection of a huge volume of blood from a large animal could be replaced by antibody isolation from egg yolk. This methodology is easier compared to the preparation of antiserum from horses. Transfer of the maternal antibody to the progeny in chickens is through the egg yolk and the antibody crosses the oviduct barrier and is termed as IgY, which accumulates in the yolk sac.

The present study describes the production of viable and safe antivenom against cobra venom using chicken egg yolk.

Lyophilized cobra venom was obtained from Irula Snake Catcher's Industrial Cooperative Society Limited, Perur P.O., Tamil Nadu. Adult inbred white leghorn hens, vaccinated against common poultry diseases were purchased from Poultry Station, Department of Animal Husbandry, TANUVAS, Chennai. Swiss albino mice and guinea pigs maintained at the animal house, King Institute of Preventive Medicine were used. Ultramembrane filters (10 and 30 kDa cut off) were purchased from Pall Life Science, Bangalore. All chemicals used were of AR/GR grade and purchased from Glaxo, Hi media, SRL and Sigma. The Institutional Animal Ethics Committee of King Institute of Preventive Medicine, Chennai, approved this study. All animals were housed in standard conditions and were provided with food and water.

White leghorn hens of strain HH 206 at the age of 15 weeks were obtained and divided into two groups. One group of four hens was primed with *Naja naja* venom in Bentonite adjuvant and boosted with neat venom. The other group was injected with normal saline as control. Hens were injected subcutaneously at multiple sites in the breast with 25–100 μg of venom. Test bleed samples were obtained by wing puncture. After satisfactory titre of antibody, the final bleed was done. Eggs were also collected, numbered and stored at 4°C. The eggs were processed according to the water dilution method of Akita and Nakai[3]. The egg was cracked, open and egg white was discarded. The egg yolk sac membrane was punctured and yolk collected. The yolk was diluted in acidified water of pH 5.5 and incubated overnight at 4°C.

The supernatant was further fractionated using 19% sodium sulphate and centrifuged at 10,000 rpm for 30 min at 4°C. The precipitate was resuspended in phosphate buffer, 12% PEG-8000 added and centrifuged. The pellet was collected and resuspended in phosphate buffer. The mixture was allowed to pass through an ultrafiltration membrane (30,000 NMWCo) to concentrate and purify the IgY antibodies. The antibodies were then filtered through 0.45 and 0.22 micron membranes to obtain sterile product.

The antibodies were analysed for protein content by Folin Ciocalteau method[4]. The specificity of IgY for venom antigen was monitored by Ouchterlony's double immunodiffusion and immunoelectrophoresis[5].

Median lethal dose (LD_{50}) of prepared cobra venom was determined in Swiss mice (18–20 g) as a multi-dose level (LD_{50}) determination by Reed and Muench method[6]. For this purpose, 10 mg/ml stock solution of cobra venom was prepared in physiological saline. 0.5 ml of each dilution of venom was injected intravenously into the mice.

*For correspondence. (e-mail: rrajendraprabhu@rediffmail.com)

The mice were observed for 24 h. 50% lethality was expressed in micrograms of venom per mouse. Control animals were injected with normal saline. The control animals survived throughout the observation period.

The double immunodiffusion (DID) plate was prepared using 1% agarose in barbiturate buffer (pH 8.6). The centre well was filled with 50 µg of cobra venom. The surrounding wells were filled with 10 µg of IgY obtained from eggs collected on day 40, 50, 60, 70 and 90. The plate was left in a humid chamber at 37°C and the precipitation bands were allowed to develop for 24 h. The gel was washed thrice with 0.15 M sodium chloride and dried. Dried gel was placed in fixative solution, preserved and documented (Figure 1).

Immunoelectrophoresis (IE) glass plate was prepared using 1.2% agarose in tris-HCl buffer (pH 8.6). A well was made using a template and filled with 10 µg of cobra venom. Electrophoresis was carried out for one hour. A trough was then made using a gel cutter and was filled with 50 µg of IgY. The plate was placed in a moist chamber to develop the precipitation arcs. The gel was washed with 0.15 M sodium chloride, fixed and documented (Figure 2).

In vivo neutralization of the lethal toxicity of the cobra venom was assessed by mixing equal volumes of 2, 5, 10 and 20 LD_{50} of cobra venom with neat and diluted IgY and incubated at 37°C for 1 h. Three mice were used for each dilution, 0.5 ml was injected intravenously per mouse. Mice were observed for 48 h for survival or death. The endpoint in potency of the product (ED_{50}) at each level was calculated by Reed and Muench method. The quantity of cobra venom, which was neutralized by 1 ml of neat and diluted IgY was calculated.

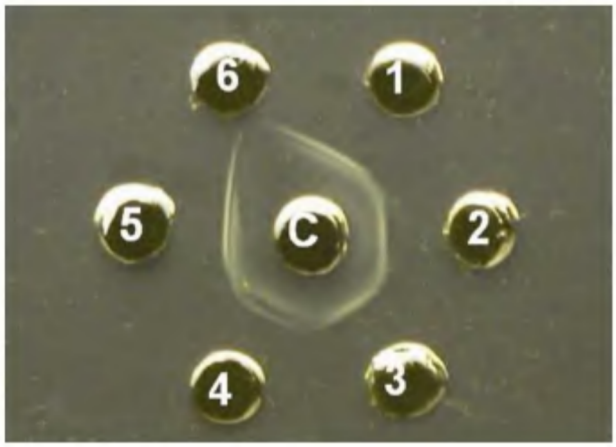

Figure 1. Detection of anti-cobra venom IgY by double immunodiffusion. Well 1: hen anti-cobra venom IgY of day 40; Well 2: hen anti-cobra venom IgY of day 50; Well 3: hen anti-cobra venom IgY of day 60; Well 4: hen anti-cobra venom IgY of day 70; Well 5: hen anti-cobra venom IgY of day 90; Well 6: un-immunized hen IgY; Well C: cobra venom 50 µg.

Undue toxicity of sterile egg yolk IgY was tested in five healthy mice and two guinea pigs as per *Indian Pharmacopoeia*[7].

The toxicity test of cobra whole venom antigen estimated the LD_{50} value as 10 µg in mice. The ability of egg yolk derived IgY, obtained from hens immunized with cobra venom to neutralize the lethal toxicity of the venom was assessed by *in vivo* potency assay. Preliminary neutralization experiments in mice indicated that each antivenom (immunized hen's serum and egg yolk IgY) was capable of neutralizing venom lethality up to 6 LD_{50}. Each egg contains approximately 100 mg of total IgY, of which the antigen-specific antibody varies from 1 to 10 mg as reported by Mary Hauk and Frendseho[8]. In this study the specific IgY antibody against cobra venom was estimated as 1.8 mg.

Detectable precipitation reaction occurred in Ouchterlony immunodiffusion after 40th day of the cobra venom immunization and strong precipitation bands appeared on the 60th day and continued up to 90th day. Control IgY did not show any precipitin line with antigen tested. Sharp precipitin lines were observed in immunoelectrophoresis with egg yolk antibodies after 40th day of immunization.

Healthy mice and guinea pigs were tested for undue toxicity. Animals observed for 7 days revealed no mortality. They did not show any sign of ill health in the 7 days following innoculation.

Antivenom/antisera production is normally carried out in large animals like horses and sheep. The method currently used by all antivenom producers throughout the world is to isolate and concentrate the antibodies from the plasma of the immunized horses and separate their enzymatically derived fragments, the $F(ab)_2$. The use of chickens for antibody production represents replacement, refinement and reduction in use of large animals.

Under standard rearing conditions, 96% livability/survivability is anticipated in experimental animals employed for antisera preparation. The various factors influencing livability are strain, housing design, climate, disease control measures, quality of feed and nature of the antigen[9]. In the present study, both groups of hens were given the same feed and indoor housing. Hence the marked increase in weight of immunized hens and its

Figure 2. Detection of anti-cobra venom IgY by immunoelectrophoresis. Well: cobra venom 10 µg; Trough: hen anti-cobra venom IgY 50 µg.

eggs compared to controls could be due to some positive factors in the cobra venom, since the P value is less than 0.01. The significance between experimental and control group of hens was at the level of 1%. The mortality level during egg laying period in 21–52 weeks of age normal hens was below 10%. The mortality during layer stage of experimental chicken was 20%. This may be due to the stress of snake venom immunization, and other endogenous factors. The mortality in the control group birds however was found to be nil.

Egg laying in hens starts in the 22nd week, and the rate of laying increases every week to reach a level of 90%, at 28 weeks of age, and continues up to 40–42 weeks of age. From the 43rd week it decreases slowly to reach less than 60% after which it is uneconomical to retain them for the production of eggs. In this study the total number of eggs was 120–130 per hen. After 52nd week the egg laying was intermittent, i.e. 2–3 eggs in fortnight and egg laying stopped after 55th week. The layer hen in the control group yielded 120–130 eggs per year. After 55th week, the egg laying stopped completely.

Fresh egg weight of immunized hens ranged from 45 to 56 g and the control hen's fresh egg weight was between 40–47 g. The weight of the egg yolk was 15–17 g in immunized group and weight of egg yolk of control was 12–15 g. All the eggs were stored for up to 1 year at 4°C prior to IgY extraction and purification.

When eggs were stored at 4°C for more than 12 months, 0.1–0.2 g loss of weight was noticed from 1st month onwards and after six months 1–2 g weight loss was observed. In the 12th month, an average of about 2–3 g loss was recorded. Thus storage for a longer period significantly affected the egg and egg yolk weight.

Soluble proteins from egg yolk were separated by 10-fold acidified water dilution method. During incubation, the lipoproteins aggregated to give a sediment. Further, salt precipitation and ultrafiltration yielded superior IgY with high specificity and having a molecular weight of 180 kDa (ref. 5). The experimental IgY passed the test for undue toxicity as prescribed in the IP[7].

Anaphylactic shock and late serum sickness reactions are attributable to the Fc portion of equine immunoglobulins. Removal of the complement reactive Fc portion of equine IgG as currently performed by enzyme digestion reduces the yield and potency of the resulting antivenom product. This procedure also adds to the manufacturing cost. In contrast, avian antivenom is clinically desirable due to the expected reduction in both the incidence of early anaphylactic and late serum sickness reactions. The procedure adopted in this study for preparation of IgY involved chemicals that are cost effective and could be easily scaled up. Twenty two chickens can produce as much immunoglobulin as a horse can yield per year[10]. The IgY in egg yolk and IgG in hen antisera forming lines of precipitation arcs after one hour of electrophoresis with cobra venom have proved and established their immune competence.

The results have demonstrated that egg laying hens respond immunologically to a wide range of antigenic components present in the cobra venom. Neutralization experiments in mice indicated that purified egg IgY after 40th day was capable of neutralizing lethality of 2 LD_{50} of venom and by the 60th day and 90th day, 5 and 6 LD_{50} respectively. The advantage of chicken IgY over mammalian antibodies is that it does not activate the mammalian complement system and does not infer with factors such as rheumatoid factors and mammalian immunoglobulins. Chicken IgY also does not bind to human or bacterial Fc receptors[2]. The present approach satisfies both scientific and commercial interests and production-wise it stands on par with the equine/ovine sources. IgY also proves to be functionally equivalent to IgG in its immunological neutralization of the snake venom antigens and its potency.

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ACKNOWLEDGEMENTS. We are grateful to Prof. G. Yogeeswaran, Dr C. S. Meenakshi and Dr K. Ramalingam, Mediclone Research Centre, for helpful discussion and valuable suggestions. Technical support by Mr S. Rameshkumar is acknowledged.

Received 26 June 2009; accepted 4 December 2009