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Research Article

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Development of red hen egg yolk antibodies against the *Naja naja* (Indian cobra) venom and its neutralisation studies

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ABSTRACT

Use of polyvalent antisera of equine has been existing as a proven treatment against snake envenomation. However it was found to be low yielding and laborious and cause many side effects. Anti-venom produced from chicken egg yolk serve an ideal alternative source to mammals, as IgY chicken's blood is transferred to the egg and accumulates in the egg yolk in large quantities. It is a safer, convenient and inexpensive method. The yolk of eggs laid by immunized chicken has been recognized as an excellent source of polyclonal antibodies. 5 month old Indian reds were immunized with cobra venom on day 0 and boosters will be given on day 14 and day 21.The presence of antibody were checked by test bleeding and thereafter eggs were collected and stored at 4˚C. Then the antibodies were extracted from egg yolk by Ammonium sulphate precipitation method. The amount of antibody produced was measured using ELISA. The potency of the produced chicken IgY antibodies was tested by neutralization studies.

Key words: Anti-snake venom, cobra, chicken, egg yolk, chicken IgY antibodies, neutralization.

INTRODUCTION

Of the over 2000 species of snakes in the world, about 200 are found in India. These ranges from the worm snakes having a length of about 10 cm to those more than 6 m long. They live in almost all habitats from the warm seas to semi-deserts, swamps, lakes and even in the Himalayan glaciers up to an elevation of about 5000 m.

The Big Four are the four venomous snake species responsible for causing the most snake bite cases in South Asia (mostly in India) namely Indian cobra (*Naja naja*, probably the most famous of all Indian snakes), Common krait (*Bungarus caeruleus)*, Russell's viper (*Daboia russelii)*, Saw-scaled viper (*Echis carinatus)*.

Snake venom is highly modified saliva usually delivered through highly specialized teeth such as hollow fangs, for the purpose of prey immobilization and self-defense. The Indian cobra's venom mainly contains a powerful post-synaptic neurotoxin and cardiotoxin. The venom acts on the synaptic gaps of the nerves, thereby paralyzing muscles, and in severe bites leading to respiratory failure or cardiac arrest. The venom components include enzymes such as hyaluronidase that cause lyses and increase the spread of the venom. Envenomation symptoms may manifest

between 15 minutes to 2 hours following the bite and can be fatal in an hour in severe cases of envenomation. The average venom yield per bite is between 169 —250 mg.

Immunotherapy is the only specific treatment against the snake's venom envenomation. (or antivenin),is a serum made from the venom of the snake. Snakebites can be extremely dangerous and serious. The venom is made up of many enzymes which help break down tissue. is needed to stop this process.Some is species specific (monovalent) while some is made for use with multiple species in mind (polyvalent). Antibodies presently available for research, diagnostic and therapies are mostly mammalian monoclonal or polyclonal antibodies. There are various side effects of using from animals, such as anaphylactic shock, pyrogen reaction and serum sickness. Most of these symptoms may be due to the action of high concentrations of non-immunoglobulin proteins present in commercially available hyper immune. [1]

Thalley and Carroll described a new avian source of s that precludes these complications and an efficient method for preparing s composed solely of venom specific antibodies.[2] Almeida *et al*., reported that adult Leghorn hens hyperimmunized with Brazilian snake venoms produced antibodies capable of recognizing, combining with and neutralizing the toxic and lethal components of the venoms.[3] Meenatchisundaram *et al*. reported that the chicken egg yolk antibodies immunoglobulin Y (IgY) were effective in neutralizing the main toxic and enzymatic effects of Cobra and Krait venoms. They also reported that the chickens could be considered as an effective alternative to mammalian antibody production in cases of diagnosis and therapy of snake bite envenomation.[4,5]

In the sense of animal protection the use of chickens for antibody production represents a refinement in that the painful collecting of blood samples and final sacrificing are replaced by collecting eggs with the synthesized antibodies in egg yolk. Since chickens produce even larger amounts of antibodies than laboratory rodents, this technology also means the reduction in the number of animals used in particular experiment.

Chicken immune system will recognize epitopes on the mammalian protein more readily and will often detect epitopes that differ from the epitopes detected by mammals, such as mice or rabbits. Keeping chickens as laboratory animals is inexpensive and requires as keeping other laboratory animals. Moreover, the active transport of IgY from serum to the egg occurs in a higher concentration than in serum. Thus, more antibodies can be produced per month than in rabbits. IgY is very stable under normal conditions. Larsson et al. mentions about stored IgY preparations for 10 years at 4°C, for 6 months at room temperature and for 1 month at 37 ˚C without any antibody loss. [6]

The production of chicken antibody brings great benefit, since the antibody preparation is a non-invasive technique making use of collected eggs. Egg yolk is an abundant and every day source of IgY (about 100 mg IgY/yolk). In addition, the isolation of IgY from egg yolk is fast and simple. Chicken antibodies also offer a lot of advantages to the common mammalian antibodies when they are used e.g. for the detection of mammalian antigen. Due to the evolutionary distance chicken IgY will react with more epitopes on a mammalian antigen, which will give an amplification of the signal. Chicken antibodies can also be used to avoid interference in immunological assays caused by the mammalian complement system, rheumatoid factor or Fc-receptors. As the antibodies can be purified in large amount from egg yolks, these immunoglobulins are suitable for passive immunization against pathogenic microorganisms and toxins. Thus, IgY technology should be considered as a good alternative and/or superior substitute to conventional polyclonal antibody production in mammals.[7]

EXPERIMENTAL SECTION

The *in vivo* studies were carried out in a Ethical committee approved institute with the permission grant number: 006/IAEC/KIPMR/2013 dated 22/10/13

2.1. Source:

2.1.1. Hens:

Two 5 months old Indian red hens weighing approximately 1kg were purchased from the Institute for Poultry Production and Maintenance (IPPM), Madhavaram, Chennai. They were kept in a individual cage with standard food and water, and all the animals were maintained with proper care. They were used in the study for the production of antisnake antibodies (IgY).

2.1.2. Venom:
Cobra venom was obtained from king's Institute for Preventive Medicine & Research, Chennai and stored at 4˚C.

2.2. Venom preparation:
1% venom -1 gram in 100ml of 3M PBS (pH:7.2) The mixture is centrifuged at 3000 rpm for 15 min.

The supernatant was collected and filtered successively through a 0.45µm and 0.22µm cellulose acetate filter membrane to get a sterile product. The filtrate is then stored at 4˚C for further use. To prepare the bentonite adjuvant/venom antigen mixture, one volume of native venom was mixed with one volume of a sterile, 2% (w/v) bentonite suspension to adsorb the venom proteins to the particulate.

2.3. Charcterization of venom:
2.3.1. Lethal dose $_{50}$
Mice weighing 18- 20 g were divided into 6 groups of 6 each.The test sample was diluted in a geometric series (usually 1.25 ml) and the volume was made up to 5 ml with the help of normal saline .Each group was injected with different concentration of venom intravenously through the tail vein.

The results were recorded 72 hrs after the injection in terms of death/live. The LD was calculated by spearmen karber method.

2.3.2. Immunization
The hens were immunized with venom (antigen) along with the adjuvant bentonite (depot) intramuscularly in the breast muscle. Chickens received subsequent booster injections with increasing concentration of venom at 14 days interval by the same route of administration. Test bleedings were made frequently to check the presence of anti venom antibodies in the serum. Eggs were collected from day 0 until the end of the experiment and stored at 4°C..
After every immunization test bleeding were done to check the presence of antibody in the blood. The blood was collected by wing puncture. The blood plasma was then separated and immunodiffusion were performed.

2.3.3. Extraction and purification of antibodies
The egg yolk was separated from egg albumen, and then rolled on the filter paper to remove white proteins. The yolk membrane was punctured and the contents was diluted by adding 9 volumes of distilled water and the pH was adjusted to 5-5.2 with 1M acetic acid and incubated at 4 ˚C for 6 to 8 hrs. Following incubation the mixture was centrifuged at 10,000 rpm for 30 min in a refrigerated centrifuge. The resulting immunoglobulins (supernatant) containing filtrates (water-soluble fraction) were collected. The supernatant was precipitated by ammonium sulphate till saturation to 60% (390g/l) the mixture was stirred at 4˚ C for 2 hrs. The precipitate was collected by centrifugation and washed with 60% saturated ammonium sulphate. The precipitate was filtered in a cheese cloth and allowed to dry. The dried product was desalted through dialysis and purified using membrane filters (0.45µm & 0.22µm). The purity of Chicken egg yolk antibodies was checked by SDSPAGE.

2.3.4. Enzyme linked immunosorbent assay:
U16 plates were coated with concentration of cobra venom and incubated at 4°C overnight. Subsequently the coated plates were washed with Phosphate Buffer Saline with Tween 20 (PBST) 4 times. The uncoated sites were blocked by 1% w/v Bovine serum albumin (BSA) (250 µl/well) and incubated at 37°C for 2 hrs. Plates were then washed and incubated with antibodies (100 µl/well) at appropriate dilutions. Wells were washed thrice with PBST and 100 µl of diluted (1:1000) rabbit. chicken immunoglobulin coupled to horse radish peroxidase was added to the well and incubated for 1 hr at 37 ˚C. The plates were then washed and 100 µl of freshly prepared substrate solution (Tetramethyl benzidine) was added to the well. The plates were allowed to stand at room temperature in the dark for 20 min.

The reaction was stopped by adding 100µl of phosphoric acid and the plates were then read at 450 nm using an ELISA reader.

2.3.5. Neutralization Efficiency
Mice weighing 18-20 g were used for the test, divided into two groups, test and control groups each group consisting of 3 animals.

Test group was injected with 1 ml of cobra venom (0.6ml of venom + 0.4 ml of normal saline) pre-incubated with 1ml of chicken IgY antibodies for 30 min at 37 ºC. Control group was injected with 1ml of diluted cobra venom alone.

The animals were kept under observation and the protective ability was measured in terms of % survival after 24 h.

RESULTS AND DISCUSSION

3.1. LD_{50} determination of venom:

The LD_{50} of the venom was determined by injecting different groups of mice with different concentration of venom. The death versus live rate was observed after 72 hrs. the LD_{50} value was calculated by Spearmen-karber method. And the LD_{50} value was found to be 9.4µg / mouse for crude venom.

3.2. Generation of antibodies in hen

The 21 week old Indian red Hens were immunized intramuscularly with prepared cobra venom to generate with two week intervals. Eggs were collected after the immunization and stored at 4°C. Then the antibodies were separated from chicken egg yolk.

3.3. Test bleeding:

After the first immunization the presence of antibodies was tested by performing immunodiffusion. The serum separated from hen's blood was used for the test. The result showed the formation of precipitin lines which confirms the presence of antibodies in hen's blood.

3.4. Isolation and purification of antibodies

Chicken egg yolk antibodies against cobra snake venom was obtained by the method of ammonium sulphate precipitation method from immunized chicken egg yolks and further purified by dialysis and membrane filters.

3.5. Protein estimation

The antibody concentration of such purified fraction after each booster dose was detected by protein estimation. The IgY concentration in the egg yolk significantly concentration of proteins increased in the egg yolk with subsequent booster doses.

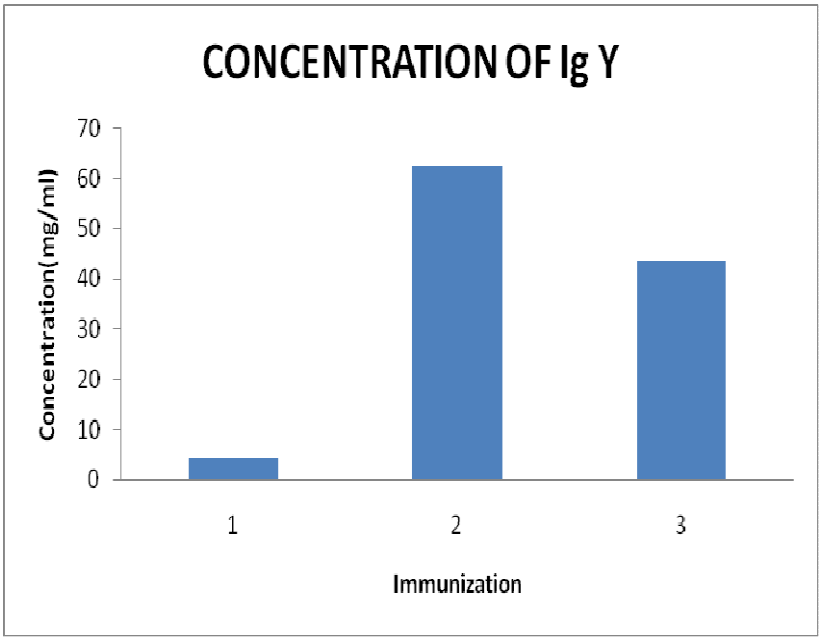

Fig 1: Protein concentration in egg

3.6. Neutralization studies: *in vitro* - antigen neutralization by ELISA

The antibody potency in neutralizing the cobra venom (antigen) was determined by ELISA. It was checked for the various dilutions of antigen and antibody.

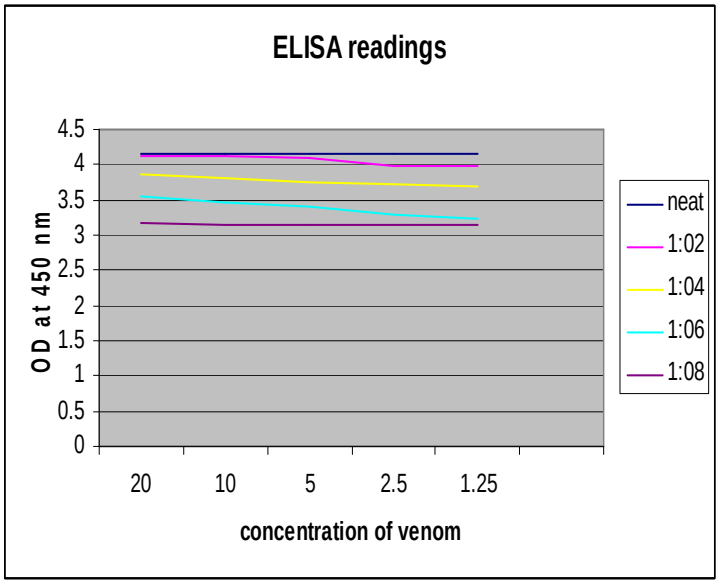

FIG 2: ELISA reading-variation in antibody titre vs dilution

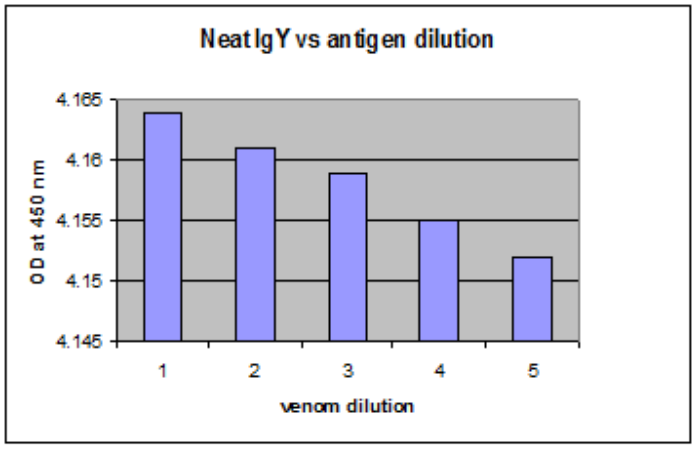

Fig 3: ELISA reading for near Ig Y and antigen dilution

The highest OD value was obtained to be 4.164µg/ml of venom. This confirms that the produced antibody can neutralize the antigen. The result showed that the antibody titer decreases with decrease in antigen concentration. The least dilution showed a titer value of 3.218 µg/ml of venom which is comparatively high.so if we even increase the dilution further, the neutralization of IgY will also be effective with minimum antigencity.

3.7. Immunodiffusion

In vitro neutralization was performed by immunodiffusion. The result of immunodiffusion showed a precipitin line which is thicker than the line produced in test bleeding. This shows that the antibody level has been increased after immunizing the hens with a total of 90µg of venom.

FIG 4 : Immunodiffusion plates

3.8. Neutralization Efficiency

The potency of the produced antibody to neutralize the venom was checked by invivo neutralization studies. This was confirmed by the neutralization studies performed in mice. All the mice were alive after injecting with the mixture of antigen and the produced chicken IgY antibody. The control group which was injected with buffer alone was found to be alive but the mice injected with increased dosage were dead. It was found that the antibody was able to neutralize 11 LD_{50} venom.

The total quantity of venom injected was 90µg which produced an average IgY concentration of 36mg/ml.Hence ammonium sulphate precipitation method and further purification steps recovered an average of 36% of IgY antibody against the cobra venom. The final protein concentration was around 5mg/ml, Further increase of antigen concentration can produce a good yield of IgY . The produced antibody was able to neutralize 11 LD_{50} of the crude venom which is higher compared to the equine antibody which can normally neutralize 5LD_{50} of the venom.

4. SUMMARY

This experiment involves production and isolation of IgY antibodies against Indian cobra venom. Cobra venom with a lethal dose of 9.4 µg/mice injected into a 5 months old chicken and boosters were given with a increasing concentration of antigen in a interval of 14 days. The eggs were collected after the immunization and stored at 4ºC.The antibodies was isolated from the immunized egg yolk using ammonium sulphate precipitation method and further purified using dialysis and membrane filters. The protein concentration of the final product obtained from egg yolk was found to be increased after each immunization. The protein concentration after the final immunization was around 5 mg/ml. the antibody titer of final IgY was determined using ELISA which showed a concentration of 4.164 g/ml.the potency of the produced IgY was tested using *in vivo* and *in vitro* neutralization studies. By *in vivo* neutralization in mice it was found that the antibody was able to neutralize 11LD_{50} of cobra venom.

CONCLUSION

The IgY antibody produced against cobra venom was extracted by ammonium sulphate precipitation method .It produced a good yield and potency. This technique can be efficiently used to treat against cobra venom which is one among the deadliest snakes commonly seen in India. It has more advantage than the mammalian antisera which are usually used for snake bite.it avoid serum reactions that normally happens in the use of mammalian antisera.

The use of chicken IgY, instead of IgG mammalian antibodies, to detect non-self or even self antigens, certainly may help lower costs of clinical or research immunological tests. In addition, chicken antibodies do not activate the mammalian complement system nor interact with rheumatoid factors, or bacterial and human Fc receptors. The advantages of chicken antibodies over mammalian antibodies include: (a) reduction in animal use, since chickens produce larger amounts of antibodies than laboratory animals; (b) the elimination of painful blood collections in animals; (c) the utility of IgY in many immunological assays without loss of specificity and sensitivity; (d) the

considerably lower cost of feeding and handling of chickens than mammalians; (e) crude egg may be used as an antibody source.

Chicken polyclonal antibodies were produced against a number of antigens and were applied in many different methods for various purposes (as a research, diagnostic, therapeutic reagents, as a tool for purification or detection of antigens and as a protective agent in passive immunization, where they provide an excellent alternative to or substitution for their mammalian counterparts. Since IgY do not react with mammalian IgG or complement system they offer a special advantage in these assays, which is reflected in great reduction of background and false results. Furthermore, the amount of antibodies produced from an egg is equivalent to that from 200 to 300 mL of mammalian blood, and the costs for animal care per unit production of antibodies are much lower in chicken than in mammals.

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