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COMPARISON OF ANTIVENOM POTENTIAL OF CHICKEN EGG YOLK ANTIBODIES GENERATED AGAINST BENTONITE AND ADJUVENT COATED VENOMES OF COMMON POISONOUS SNAKE IN INDIA

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ABSTRACT

Antivenom antibodies were generated in white leghorn chicken against bentonite and adjuvant coated venoms of Common Indian Poisonous Snakes (Cobra, Krait, Russell's viper and Saw Scaled viper).The antivenom from immunized chicken egg yolk were purified by polyethylene glycol (PEG) and ammonium sulphate precipitation method and further purified by DEAE cellulose ion exchange column chromatography and concentrated by polyvinyl pyrolidone powder at room temperature. The titer of antibodies was estimated using Enzyme Linked Immunosorbent Assay (ELISA).The chickens immunized with Freund's complete adjuvant showed slightly higher titre when compared to bentonite. Inhibition of lethal, edema, haemorrhagic, procoagulant and phospholipase A_2 and fibrinolytic activities of snake venoms were determined. The chicken egg yolk antivenom was effective in neutralization of these toxic and enzymatic activities of venom. The median effective dose (ED_{50}) of chicken egg yolk antibodies raised against adjuvant coated venoms showed effective neutralizing venom toxicity when compared to the antibodies raised using bentonite coated venoms.

Key words: Venom, Chicken antibodies (IgY), edema, PLA_2

INTRODUCTION

Snake envenoming is a major public health issue in the rural tropics with large numbers of envenoming and deaths. There are nearly 3000 different species of snakes found in the world of which approximately 300 are venomous. In India there are about 216 different species are found, of which 53 species are reported to be poisonous (Whitaker, 1978). The common poisonous snakes found in India are Cobra, Krait, Russell's viper and Saw Scaled Viper (Bawaskar, 2004). About 35,000 to 50,000 people die of snake bite every year in India (Sharma *et al.*, 2004). Antivenom immunotherapy is the only specific treatment against snake venom envenomation. Antivenom is the serum that is commercially produced to neutralize the effects of envenomation by venomous snakes. Commersially available Horse antivenom contained high concentrations of non-immunoglobulins which frequently caused complement mediated side effects, serum sickness and renal failure which may be reduced by using sufficiently pure antibodies. In India polyvalent antisnakevenom effective against venoms of cobras, krait, Russell's viper and saw-scaled viper is available. Each mililitre of polyvalant antisnake venom can neutralise 0.6 mg of Cobra, 0.6 mg of Russell's viper, 0.45 mg of Krait and 0.45 mg of Saw scaled viper venom. Antivenoms may be species specific (monovalent) or effective against several species (polyvalent). Monovalent antivenom is ideal (Philip, 1994), but the cost and non-availability, besides the difficulty of accurately identifying the offending species - makes its use less common (Warrel, 1996). Biotechnology companies are working on a new generation of purer anti-venoms, which should be safer. Recently Thalley and Carroll (1990) described a new avian source of antivenoms that precludes these complications and an efficient method for preparing antivenoms composed solely of venom specific antibodies. Almeida *et al*. (1998) reported that adult white leghorn hens hyperimmunized with Brazilian snake venoms produced antibodies capable of recognizing, combining with and neutralizing the toxic and lethal components of the venoms. Meenatchisundaram *et al*. (2008) reported that the chicken egg yolk antibodies (IgY) were effective in neutralizing the main toxic and enzymatic effects of Cobra and Krait venoms. He 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. The present study involves the comparison of the effectiveness of Freund's complete adjuvant and bentonite adjuvant in generation of antivenom against the four common poisonous snakes of India and the ability of these antibodies in neutralizing the various pharmacological activities induced by snake venoms by both *in vivo* and in vitro methods.

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MATERIALS AND METHODS

Venom and experimental animals

The freeze-dried snake venom powders of *Naja naja, Bangarus caeruleus*, *Dabioa russelli* and *Echis carinatus* were obtained from Irula's Snake Catchers Industrial Co-operative Society Limited, Chennai and was stored at 4°C. Twenty four week old, single comb white leghorn chickens obtained from the Abinaya Poultry Farm, Namakkal and were maintained in our animal facility. They were used in the study for the production of antivenom (IgY). Male inbreed Swiss Albino mice of 18-20 gm body weight were used for the studies of venom toxicity and in the experiments of venom neutralization. Institutional Animal Ethics Committee clearance at Institute of Vector Control and Zoonoses, Hosur, was obtained to conduct the experiment. All the animals were conditioned in standard cages.

Development of antivenom antibodies in chicken

The lyophilized snake venom powder of Cobra, Krait, Russell's viper and Saw Scaled viper venom were dissolved separately in 0.9% phosphate buffered saline (PBS) in the concentration of 1mg/ml. The diluted snake venoms were then centrifuged and filter sterilized to remove the impurities. The protein concentrations of snake venoms were estimated by the method of Lowry (1951). To prepare the Freund's adjuvant/venom antigen mixture, 50 µl of venom mixed and emulsified with Freund's Complete Adjuvant (FCA) in the ratio of 1:1 using the technique of Herbert (1967). 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 (Sigma) suspension to adsorb the venom proteins to the particulate. Then the solution was injected subcutaneously into multiple sites of the breast muscles into six white leghorn hens on day zero. Three birds received the antigen with FCA. The remaining three birds received the antigen absorbed to bentonite. Test bleedings were made frequently to check the presence of antivenom antibodies in the serum. Eggs were collected from day 0 until the end of the experiment and stored at 4°C until testing by the indirect ELISA.

Purification and Characterization of antivenom antibodies from egg yolk

The antibodies were extracted from egg yolk by the method of Polson *et al*. (1980) using Polyethylene and Ammonium sulphate precipitate method. Then the content was desalted by dialysis process. The crude fraction of IgY thus obtained was further purified by DEAE cellulose ion exchange column chromatography. The IgY fraction was then concentrated with Poly Vinyl Pyrolidone (PVP) at room temperature. The protein content of the purified IgY fraction was determined by the method described by Lowry *et al*. (1951).

Determination of antibody titer by Indirect ELISA

The antibody titer of the antibodies generated against Cobra, Krait, Russell's viper and Saw Scaled viper venoms were determined by indirect ELISA described by Voller *et al*. (1976). Nunc polysorp ELISA plates were coated with snake venoms at a concentration of 1µg / 100µl / well using coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. After coating plates were washed with PBST for 3 times. The empty sites blocked by 1% BSA (200 µl / well) and incubated at 37°C for 1 hour. Plates were subsequently washed and incubated with antivenom antibodies (100 µl / well). PBST and preimmune sera served as controls. Wells were washed thrice with PBST and 100 µl of diluted (1:1000) rabbit antichicken immunoglobulin coupled to Horse Radish Peroxidase (Genei Pvt Ltd, Bangalore) was added and incubated. Then the plates were washed and 100 µl of freshly prepared substrate solution (4 mg of O-phenylene diamine dissolved in 10 ml of 50 mM citrate buffer, pH 5.0 containing 10 µl of 30% hydrogen peroxide) was added. The plates were allowed to stand at room temperature in dark for 20 minutes. The reaction was stopped by adding 50 µl of 4N Sulphuric acid and plates were read at 490 nm in an ELISA reader. All samples were tested in triplicates.

In vivo assessment of venom toxicity and anti-venom activity of egg yolk antibodies

Lethal toxicity

The median lethal dose (LD_{50}) of Cobra, Krait, Russell's viper and Saw Scaled viper venoms were determined according to the method developed by Theakston and Reid (1983). Various doses of venom in 0.2 ml of physiological saline was injected into the tail vein of mice (18-20 gms), using groups of 3-5 mice at each venom

dose. The LD_{50} was calculated with the confidence limit at 50% probability by the analysis of deaths occurring within 24 hrs. of venom injection. The anti-lethal potentials of chicken egg yolk antibodies were determined against $2LD_{50}$ of snake venoms. Various amount of IgY were mixed with $2LD_{50}$ of venom sample and incubated at 37°C for 30 minutes and then injected intravenously into mice. Three to Five mice were used at each antivenom dose. Control mice received same amount of venom without antivenom. The median Effective Dose (ED_{50}) calculated from the number of deaths within 24 hrs. of injection of the venom/antivenom mixture. The ED_{50} was expressed as µl antivenom/mouse and calculated by probit analysis (XL Stat 2008).

Edema- forming Activity

The minimum edema-forming dose (MED) of Cobra, Krait, Russell's viper and Saw Scaled viper venoms were determined by the method of Lomonte *et al*. (1993) and Camey *et al*. (2002). The minimum edema-forming dose (MED) was defined as the least amount of venom which when injected subcutaneously into mice footpad results in 30% edema within 6 hours of venom injection. The thickness of each footpad was measured every 30 min after venom injection with a low-pressure spring caliper (Rojas *et al*., 2005). The ability of chicken egg yolk antibodies in neutralizing the edema- forming activity were carried out by pre-incubating the constant amount of venom and various dilutions IgY and incubated for 30 minutes at 37°C. Then, groups of four mice (18 – 20 g) were injected subcutaneously in the right footpad with 50 µl of the mixtures, containing venom / IgY, whereas the left footpad received 50 µl of PBS alone. Control mice were injected with venom in the right footpad and 50 µl of PBS in the left footpad. One hour after injection edema was evaluated as described by Yamakawa *et al*. (1976). Edema was expressed as the percentage increase in thickness of the right footpad compared to the right footpad of the control mice.

Haemorrhagic activity

The minimum haemorrhagic dose (MHD) of Cobra, Krait, Russell's viper and Saw Scaled viper venoms were determined by the method described by Theakston and Reid (1983). The minimum haemorrhagic dose was defined as the least amount of venom which when injected intradermaly (i.d.) into mice results in a haemorrhagic lesion of 10 mm diameter in 24 hours. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts IgY. The IgY venom mixture was incubated at 37°C for 1 hr. and 0.1 ml of the mixture was injected intradermaly into mice. The haemorrhagic lesion was estimated after 24 hrs.

In vitro assessment of venom toxicity and anti-venom activity of egg yolk antibodies

Phospholipase activity

Phospholipase A2 activity was measured using an indirect hemolytic assay on agarose–erythrocyte–egg yolk gel plate by the methods described by Gutierrez *et al*. (1988). Increasing doses of Cobra, Krait, Russell's viper and Saw Scaled viper venoms (µg) was added to 3 mm wells in agarose gels (0.8% in PBS, pH 8.1) containing 1.2% sheep erythrocytes, 1.2% egg yolk as a source of lecithin and 10 mM calcium chloride. Slides were incubated at 37˚C overnight and the diameters of the hemolytic halos were measured. Control wells contained 15 µl of saline. The minimum indirect hemolytic dose (MIHD) corresponds to a dosage of venom, which produced a hemolytic halo of 11 mm diameter. The efficacy of IgY in neutralizing the phospholipase activity was carried out by mixing constant amount of venom (µg) with different amount of IgY (µl) and incubated for 30 minutes at 37°C. Then, aliquots of 10µl of the mixtures were added to wells in agarose-egg yolk-sheep erythrocyte gels. Control samples contain venom without antibodies. Plates were incubated at 37°C for 20 hours. Neutralization expressed as the ratio mg antibodies/mg venom able to reduce by 50% the diameter of the hemolytic halo when compared to the effect induced by venom alone.

Procoagulant activity

The procoagulant activity was done according to the method described by Theakston and Reid (1983) modified by Laing *et al*. (1992). Various amounts of venom dissolved in 100 µl PBS (pH 7.2) was added to human citrated plasma at 37°C. Coagulation time was recorded and the Minimum Coagulant Dose (MCD) was determined as the venom dose, which induced clotting of plasma within 60 seconds. Plasma incubated with PBS alone served as control. In neutralization assays constant amount of venom was mixed with various dilutions of chicken egg yolk antibodies. The mixtures were incubated for 30 minutes at 37°C. Then 0.1 ml of mixture was added to 0.3 ml of

citrated plasma and the clotting times recorded. In control tubes plasma was incubated with either venom alone or IgY alone. Neutralization was expressed as effective dose (ED), defined as the ratio µl antivenom (IgY) / mg venom at which the clotting time increased three times when compared with clotting time of plasma incubated with two MCD of venom alone.

Fibrinolytic activity

A modified plaque assay was used (Rojas *et al*., 1987). The minimum fibrinolytic concentration was defined as the concentration of venom that induced a fibrinolytic halo of 10mm diameter. Neutralization experiments were performed by incubating a constant amount of venom with varying amount of IgY at 37°C for 1 hr. After incubation, the mixture was applied to the wells in the plaque. After 18 hrs. of incubation at 37°C, fibrinolytic halos were measured.

Statistical analysis

Statistical evaluation was performed using XL stat 2007 and SPSS 10 Softwares.

RESULTS AND DISCUSSION

Generation of snake antivenom antibodies in chicken

White leghorn chickens were immunized intramuscularly with sublethal dose of Cobra, Krait, Russell's viper and Saw Scaled viper venoms emulsified with Freund's Complete Adjuvant in one sets of chicken and bentonite coated snake venom antigens in another sets of chickens for the generation of antivenom antibodies. The preimmune sera and hyperimmune sera were collected at specified time intervals during and after the various immunization schedules. Then specific antibodies were detected in egg yolk after a week. The antibodies were extracted by ammonium sulphate and Polyethylene Glycol (PEG) precipitation methods and further purified by DEAE Cellulose Ion Exchange Column Chromatography. The protein content egg yolk antibodies generated against adjuvant (FCA) coated snake venoms varied from 5 - 6.65mg/ml and antibodies raised against bentonite coated snake venoms varied from 0.4 - 5.96 mg / ml.

Adjuvant effects on antivenom titers

In ELISA there was a gradual increase in the antibody titer in both egg yolk antibodies (FCA and Bentonite) and reached a pleatue and remained stable till 180th day of observation. The booster doses administered at regular time intervals increased and maintained the antivenom level in yolk. The birds immunized with freunds adjuvant coated venoms showed very high titer at dilutions of more than 1:10000 detecting even less than 0.080 µg of specific antivenom (Fig. 1). The birds immunized with bentonite venoms also showed good titre value but lesser than FCA. All the birds show a significant titer as compared with the unimmunized control. The chickens immunized with FCA coated venoms did not produce any side effects in birds but bentonite caused a decrease in the laying frequency of the bird. These side effects caused by bentonite may lead to decrease in antivenom titres in egg yolk of immunized hens.

Neutralization assays

The antivenom potential of chicken egg yolk antibodies generated against bentonite and adjuvant (FCA) coated Cobra, Krait, Russell's viper and Saw Scaled viper venoms were tested by in vivo and in vitro methods. The lethal toxicity (LD_{50}) Cobra, Krait, Russell's viper and Saw Scaled viper venoms were assessed using 18 g, Balb / c strain mice. The LD_{50} of venom for 18 g of mice was found to be 10 µg for Cobra, 3 µg for Krait, 8 µg for Russell's viper and 12 µg for Saw scaled viper venom. The neutralization of lethality was done by mixing constant amount of venom with various dilutions of chicken egg yolk antibodies and incubated at 37°C for 30 minutes prior to injection. We found that chicken egg yolk antibodies generated using FCA coated venoms showed better results than bentonite coated venoms (Table 1).

In edema forming activity, the mice immunized with Cobra, Krait, Russell's viper and Saw Scaled viper venoms were showed increase in footpad thickness. About 5 µg of Cobra, 2 µg of Krait, 5 µg of Russell's viper and 7 µg of Saw scaled viper venom induced edema formation within 3hrs. which is considered as 100 % activity. The edema was reduced up to 30 % when 3500 µl of IgY / mg venom was given. There was no further reduction in the percentage of edema even when there was an increase in antivenom dose (Fig. 2).

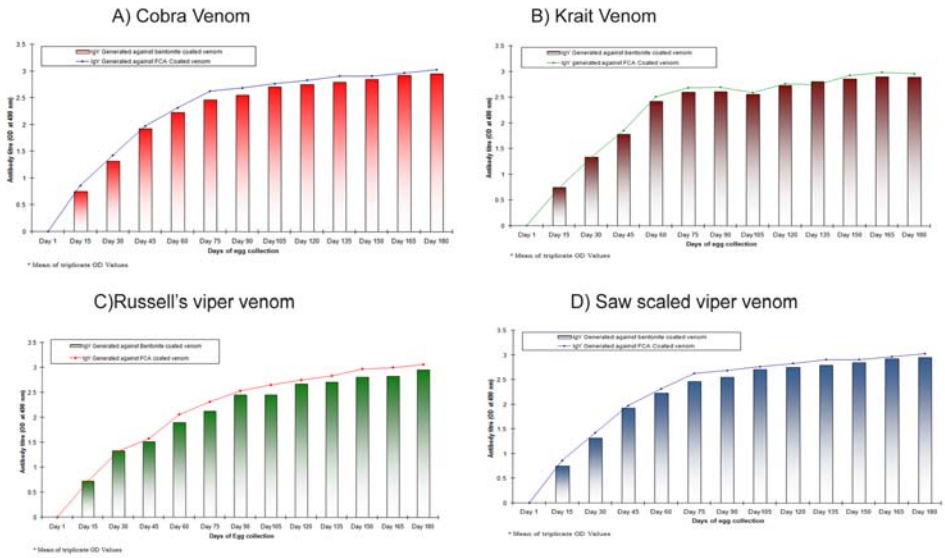

Fig. 1. Kinetics of antibody production in hens immunized with Cobra, KRAIT, Russell's viper and Saw scaled viper venoms (A, B, C & D). The titre of antivenom in chicken egg yolk against bentonite and FCA were determined by ELISA. The chicken immunized with freunds adjuvant coated venoms (FCA) showed very high titer when compared to antibodies generated using bentonite coated venoms

Table 1. Neutralization of snake venom induced lethality by chicken egg yolk antibodies

Venoms	Dose of venom (µg)	Neutralization of venom by *Egg yolk antibodies* (ED_{50} in mg)	
		FCA	Bentonite
Cobra	20 ($2LD_{50}$)	1.27	1.33
Krait	6 ($2LD_{50}$)	1.14	1.26
Russell's viper	16 ($2LD_{50}$)	1.21	1.25
Saw scaled viper	24 ($2LD_{50}$)	1.24	1.28

Neutralization of edema by chicken egg yolk antibodies generated against adjuvant (FCA) and bentonite coated Cobra, Krait, Russell's viper and Saw scaled viper venoms. Various mixtures of snake venoms and chicken egg yolk antibodies were incubated and tested in the footpad assay. Edema was assessed 1 hr. after injection and expressed as percentage. Edema induced in control mice (venom alone) was considered as 100% activity. Both chicken egg yolk antibodies inhibited the edema forming activity and edema was reduced upto 30% (Fig. 2).

In the case of hemorrhagic activity, only *Echis carinatus* venom produced visible hemorrhagic spot and both chicken egg yolk antibodies were effectively neutralized *Echis carinatus* venom induced hemorrhagic activity. In phospholipase activity (PLA_2), all snake venoms able to produce hemolytic haloes in agarose-sheep erythrocytes gels. Both chicken egg yolk antibodies were capable of inhibiting PLA2 dependent hemolysis of sheep RBC's induced by snake venoms in a dose dependent manner (Table 2).

A) B)

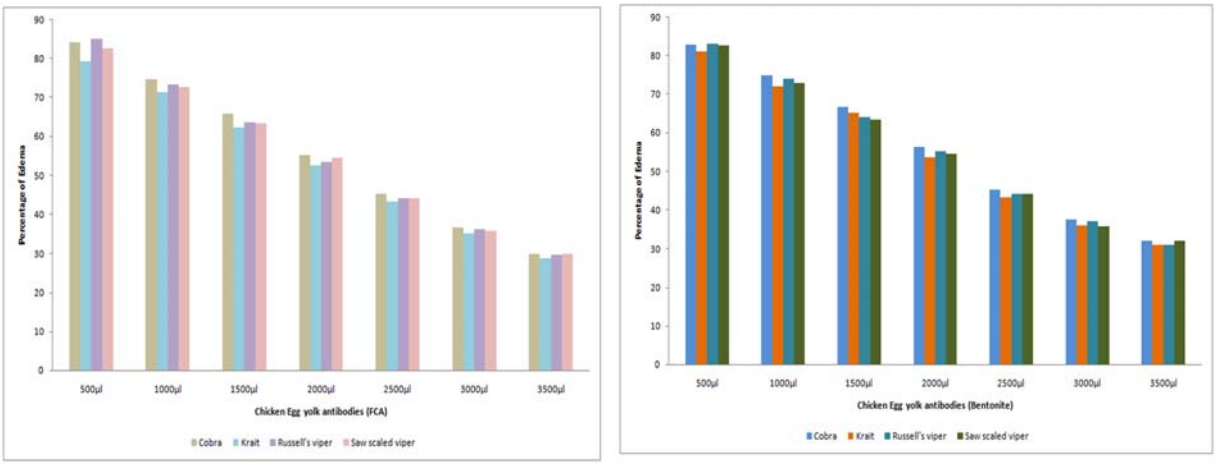

Fig.2. Neutralization of edema by chicken egg yolk antibodies generated against adjuvant (FCA) and bentonite coated Cobra, Krait, Russell's viper and Saw scaled viper venoms.

Table 2. Phospholipase activity of snake venoms and its neutralization by chicken egg yolk antibodies

Venoms	Dose of venom (µg)	Neutralization of venom by *Egg yolk antibodies* (ED_{50} in mg)	
		FCA	Bentonite
Cobra	15 (1 Unit)	1.21	1.25
Krait	10 (1 Unit)	1.08	1.14
Russell's viper	15 (1 Unit)	1.15	1.21
Saw scaled viper	10 (1 Unit)	1.17	1.23

The minimum coagulant dose (MCD) was determined as the venom concentration inducing clotting of plasma in 60s. About 40 µg of Cobra and Krait and 120 µg of Russell's viper and Saw scaled venom clotted human citrated plasma within 60s. In the neutralization assay, the absence of clot formation shows the neutralizing ability of both chicken egg yolk antibodies (Table 3). The fibrinolytic effect was effectively antagonized by chicken egg yolk antibodies generated using bentonite and FCA coated venoms.

Snakebite is a common medical emergency encountered in the tropics and estimated 35,000 to 50,000 people die of snakebite every year in India (Sharma *et al.*, 2004). Antivenom is the specific antidote for snakebite envenomation. The commercial antivenom consists of polyclonal antibodies produced by fractionating blood from horses immunized with venom (Hill *et al.*, 2001). Antisnake venom therapy may cause various side effects auch as anaphylactic shock, pyrogen reaction and serum sickness (Maya Devi *et al.*, 2002). In recent years, immunoglobulins obtained from avian egg yolks are increasingly finding favour to replace mammalian antibodies for diagnostic and therapeutic applications.

Table 3. Procoagulant activity of snake venoms and its neutralization by chicken egg yolk antibodies

Venoms	Dose of venom (µg)	Neutralization of venom by *Egg yolk antibodies* (ED_{50} in mg)	
		FCA	Bentonite
Cobra	40	1.35	1.43
Krait	40	1.26	1.35
Russell's viper	120	1.45	1.51
Saw scaled viper	120	1.48	1.55

Hens produce a more hygienic, cost efficient, convenient and a plentiful source of antibodies, as compared to traditional method of obtaining antibodies from mammalian serum (Gassmann *et al.*, 1990). The present investigation was carried out to raise specific polyclonal hyper-immune antibodies in chicken against bentonite and adjuvant (FCA) coated Cobra, Krait, Russell's viper and Saw Scaled viper venoms. Snake venom antigens were immunized intramuscularly at multiple sites of breast muscles. The presence of IgY in the yolk is detected four to seven days after appearance in serum. The concentration of antibodies increased in the egg yolk with subsequent booster doses with an average yield of 80mg per egg yolk at 180th day of immunization period. The ELISA there was a gradual increase in the antibody titer in both egg yolk antibodies (FCA and Bentonite) and reached a pleatue and remained stable till 180th day of observation. The birds immunized with Freunds adjuvant coated venoms showed very high titer at dilutions of more than 1:10000 detecting even less than 0.080 µg of specific antivenom. The chickens immunized with FCA coated venoms did not produce any side effects in birds but bentonite caused a decrease in the laying frequency of the bird. These side affects caused by bentonite may lead to decrease in antivenom titres in egg yolk of immunized hens. Rungsiwongse and Ratanabanangkoon (1991) reported a significant correlation between their ELISA and the antivenom potency tested in vivo against Naja naja venom. Chickens are obtainable in inbred strains thus minimizing the genetic variation in antibody response, also providing much more hygienic, cost efficient, convenient, humane and plentiful source of antigen specific antibodies. In this several international organizations such as WHO, FDA(USA) and the European Centre for the Validation of Alternative methods (ECVAM, European union) have recently recommended the reduction of animal testing and emphasized the importance of diminishing the pain inflicted to them (Theakston *et al*., 2003). It is essential to understand the pharmacological action of snake venom in order to device a rational treatment for snakebite. The neutralization studies were carried out to find out the efficiency of IgY antibodies against toxic components of venom. Neutralization studies can be performed by incubating of venom and chicken egg yolk antibodies prior to testing (pre-incubation method). The results showed that the both chicken egg yolk antibodies were capable of neutralizing the lethality induced by the venom. The variations in the ED50 values between venoms are due to the antigenic differences between toxins present in the venoms. The neutralization ability of snake antivenoms is still assessed by traditional in-vivo lethality assay [median effective dose (ED50)], comparable to those used for bacterial antitoxins, usually performed in mice (WHO,1981). Both chicken egg yolk antibodies were capable of inhibiting PLA2 dependent hemolysis of sheep RBCs in a dose dependent manner. The antivenom antibodies from chicken egg yolk recognized the snake venom component, which has biological activities like phospholipase activity (Almeida et al., 1998). The antivenom potency correlated significantly with inhibition of PLA2 activity (Maria et al., 1998).

Edema-forming activity was assessed for all snake venoms and both chicken egg yolk antibodies were found to be effective in neutralization of edema induced by venoms. There was a significant decrease in the edema (footpad thickness) when there was an increase in the antivenom concentration. Procoagulant activity induced by snake venoms were studied using human citrated plasma and both chicken egg yolk antibodies were found to be effective in the neutralization of procoagulant activity. Camey *et al*. (2002) worked on Pharmacological characterization and neutralization of venoms used in the production of Bothropic antivenom in Brazil and reported that the Brazilian antivenom is effective in neutralizing the main toxic effects of Bothrops venoms. Previous research showed that purified avian C. atrox and T. Flavoviridis chicken antivenoms were 6.3 and 2.0

times as potent as equine antivenom in neutralizing the venom lethality (Thalley *et al*., 1990). The present experimental results indicate that both chicken egg yolk antibodies were effective in neutralizing the main toxic and enzymatic effects of Cobra, Krait, Russell's viper and Saw Scaled viper venoms. In conclusion, Freund's adjuvant works well without causing any side effects to birds and the antibody production also good. Based on the results it is confirmed that Freund's adjuvant is the preferred adjuvant to generate chicken egg yolk antivenom antibodies to treat snake bite envenomations.

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