Short Communication

Comparison of four different purification methods for isolation of anti *Echis carinatus* antivenom antibodies from immunized chicken egg yolk

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Abstract
Egg-laying hens were immunized with the *Echis carinatus* venom and the resulting antibodies were extracted from egg yolk by four different purification methods. The chicken egg yolk antibodies were purified by the water dilution method, polyethylene glycol (PEG) and ammonium sulphate precipitation method, chloroform extraction and the Lithium sulphate precipitation method. These methods were compared in terms of total protein content, immunospecific anti *E. carinatus* immunoglobulin Y (IgY) activity and *in vitro* and *in vivo* neutralizing capacity of IgY against the *E. carinatus* venom. Total IgY concentrations varied from 1.6 to 7.0 mg per ml of egg yolk. In neutralization studies, IgY purified by PEG and ammonium sulphate precipitation (PEG-AS) showed better results when compared to other purification methods. Approximately 1.25 mg of IgY (PEG-AS) was able to neutralize 2Lethal $Dose_{50}$ of the *E. carinatus* venom. Purification of IgY by PEG and ammonium sulphate yielded very pure IgY at high quantities (93% ± 5% of total egg yolk protein), which was also capable of neutralizing toxic and lethal components of the *E. carinatus* venom.
Key words: Venom; IgY; Lethality; Haemorrhagic; PLA_2

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Snakebite is a major health hazard that leads to high mortality rates, especially in India. The common poisonous snakes found in India are Cobra (*Naja naja*), Krait (*Bangarus Caeruleus*), Russell's viper (*Daboia russelli*) and the Saw Scaled Viper (*Echis Carinatus*) (Bawaskar, 2004). Approximately 35,000 to 50,000 people die of snakebite every year in India. The venoms of Cobra and Krait of the Elapidae family are neurotoxic in nature, which are thought to attack the victim's central nervous system, usually resulting in heart failure. Their venom constitutes three types of proteins namely cardiotoxins, neurotoxins and Phospholipase A_2, which are responsible for various toxic activities in patients with snakebite.

Antivenom immunotherapy is the only specific treatment against the snake's venom envenomation. There are various side effects of using antivenom, 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 antivenom (Devi *et al*., 2002). 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 a,b) 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. 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.

In the present study, antibodies were generated in chicken against the *E. carinatus* venom. The antibodies were extracted from egg yolk and purified by four different purification methods. These antibodies were compared in terms of total protein content, immunospecific anti *E. carinatus* IgY activity, *in vitro* and *in vivo* neutralizing capacity of IgY against the *E. carinatus* venom.

The freeze-dried snake venom powder of *E. carinatus*, obtained from Irula's Snake Catchers Industrial Co-operative Society Ltd in Chennai, India, was stored at 4ºC. Twenty four week old, single comb white leghorn chickens obtained from the Abinaya poultry farm in Namakkal, India were maintained in the animal facility at PSG College of Arts and Science, Coimbatore. They were used in the study for the production of antivenom antibodies (IgY). Male inbreed Swiss albino mice (weighing 18-20 g) were used in the studies of venom toxicity and neutralization. An institutional animal ethics committee clearance at the Institute of Vector Control and Zoonoses (IVCZ), Hosur, was obtained to conduct the experiment. All the animals were conditioned in standard cages.

The lyophilized snake venom powder of *E. carinatus* was dissolved in 0.9% phosphate buffered saline (PBS), at the concentration of 1 mg/ml. The diluted venom samples were then centrifuged and filter sterilized to remove impurities. The protein concentration of the venom was estimated by the method of Lowry *et al*., (1951). Approximately 50 µl of venom was mixed and emulsified with Freund's complete adjuvant (FCA), at the ratio of 1:1, using the technique of Herbert (1967). The resulting solution was then injected subcutaneously into multiple sites of the breast muscles of white Leghorn hens on day zero. Chickens received subsequent booster injections with increasing concentrations of venom at 14-days intervals by the same route of administration. Bleeding tests were made frequently to check the presence of antivenom antibodies in the serum. Eggs were collected from day zero until the end of the experiment and stored at 4°C until testing by the indirect ELISA method. The yolks of five eggs were collected 11 weeks after the second immunization and separated from the egg whites, then washed with distilled water to remove as much albumin as possible, and rolled on paper towels to remove adhering egg white. The yolks were pooled, mixed and aliquots of these mixture were then processed according to four different protocols. 1) water dilution method (Akita and Nakai, 1992) 2) Polyethylene glycol (PEG) and ammonium sulphate precipitation method (Polson *et al*., 1980) 3) chloroform extraction method (Ntakarutimana *et al*., 1992) and 4) lithium sulphate precipitation method (Bizhnow *et al*., 2004). The antibodies were further purified by DEAE cellulose ion exchange column chromatography. The IgY fraction was then concentrated with polyvinylpyrrolidone (PVP) at room temperature. The concentrated

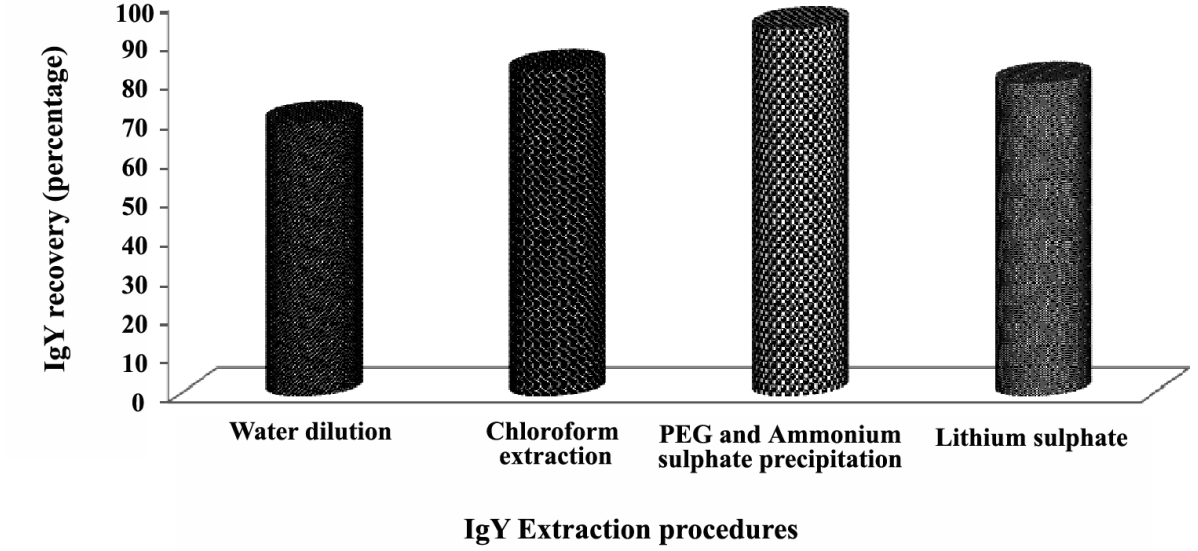

Figure 1. Compartitive analysis of anti *E. carinatus* antibodies (IgY) purified by four different purification methods.

protein level was then checked as mentioned above.

The antibody titre against the *E. carinatus* venom was determined by the indirect Enzyme Linked Immunosorbant Assay (ELISA) method described by Voller *et al*. (1976). Nunc polysorp plates were coated with the venom at a concentration of 1 µg/100 µl/well using coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) and incubated at 4°C overnight. Subsequently the coated plates were washed with Phosphate Buffer Saline with Tween 20 (PBST) 3 times. The uncoated sites were blocked by 1% w/v Bovine serum albumin (BSA) (200 µl/well) and incubated at 37°C for 1 h. Plates were then washed and incubated with antivenom antibodies (100 µl/well) at appropriate dilutions. PBST and preimmune sera were used 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, India) was added to the well and incubated. The plates were then washed and 100 ml of freshly prepared substrate solution (4 mg of O-phenylene diamine dissolved in 10ml of 50 mM citrate buffer containing 10 ml of 30% hydrogen peroxide, pH 5.0) 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 50 ml of 4 N H_2SO_4 and the plates were then read at 490 nm using an ELISA reader. All samples were tested in triplicates.

The median lethal dose (LD_{50}) of the *E. carinatus* venom was determined according to the method developed by Theakston and Reid (1983). Various doses of venom in 0.2 ml of physiological saline were injected into the tail vein of mice using groups of 5 mice at each venom dose. The LD_{50} was calculated with the confidence limit at 50% probability by scoring deaths occurring within 24 h of venom injection. The anti-lethal potentials of chicken egg yolk antibodies were determined against 2 Lethal $Dose_{50}$ of the venom. Various amounts of IgY (80 µl-200 µl) were mixed with $2LD_{50}$ of the venom sample and incubated at 37 ºC for 30 min, then injected intravenously to mice. Each antivenom dose was injection to 3-5 mice. Control mice received 80 µl-200 µl of venom without antivenom. The median effective dose (ED_{50}) was calculated from the number of deaths within 24 h of injection of the venom/antivenom mixture. The ED_{50} was expressed as µl of antivenom/mouse and calculated by probit analysis. The minimum haemorrhagic dose (MHD) of the *E. carinatus* venom was determined by the method described by Theakston and Reid (1983) and is defined as the least amount of venom, which when injected intradermally (i.d.) into mice results in a haemorrhagic lesion with a 10 mm diameter in 24 h. Neutralization of the haemorrhagic activity was estimated by mixing a fixed amount of venom with different amounts of egg yolk antibodies. The IgY-venom mixture was incubated at 37°C for 1 h and 0.1 ml of the mixture was then injected intradermally into mice. The haemorrhagic lesion was estimated after 24 h. Phospholipase A2 activity was measured using an indirect haemolytic assay on an agarose-erythrocyte-egg yolk gel plate by the method described by

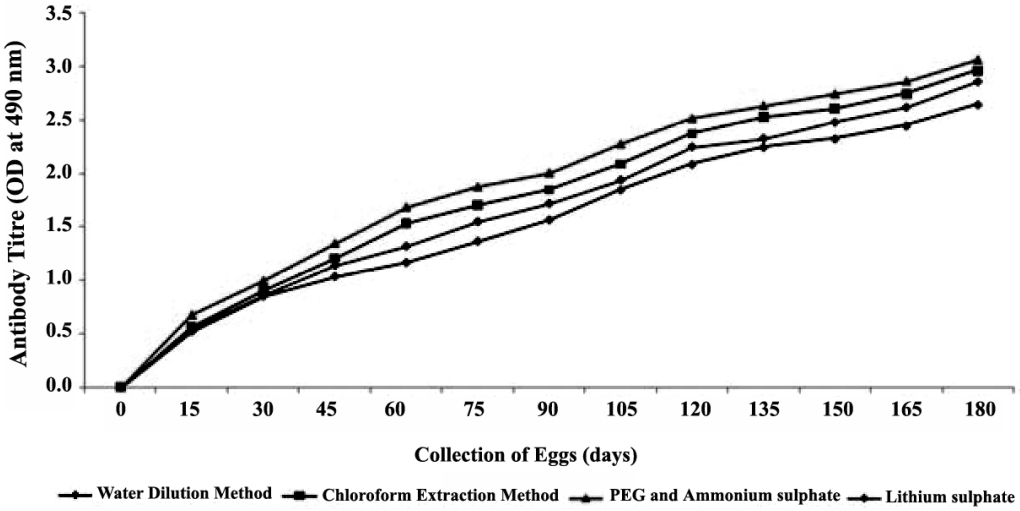

Figure 2. Kinetics of antibody production in hens immunized with the *E. carinatus* venom. The titer of antivenom in immunized chicken egg yolk was estimated by ELISA. There was a gradual increase in antibody titer with subsequent booster dose administrations.

Gutierrez *et al*. (1988). Increasing doses of the venom (µg) were added to 3 mm wells in the agarose gel slides (0.8% w/v in PBS, pH 8.1) containing 1.2% v/v sheep erythrocytes, 1.2% v/v egg yolk as a source of lecithin and 10 mM $CaCl_2$. Slides were incubated at 37°C overnight and diameters of the resulting haemolytic halos were then measured. Control wells contained 15 µl of saline. The minimum indirect haemolytic dose (MIHD) corresponds to a dosage of venom, which produces a haemolytic halo, 11 mm in diameter. The efficacy of the antivenom (IgY) in neutralizing the phospholipase activity was carried out by mixing constant amounts of venom (µg) with different amounts of IgY (µl), followed by incubation for 30 min at 37°C. Then, 10 ml aliquots of the mixtures were added to wells in the agarose-erythrocyte-egg yolk-gel plate. Control samples contained venom without IgY. Plates were then incubated at 37°C for 24 h. Neutralization was expressed as the ratio of mg antibodies/mg venom able to reduce by 50% the diameter of the haemolytic halo when compared to that induced by venom alone. The procoagulant activity was carried out according to the method described by Theakston and Reid (1983) and modified by Laing *et al*. (1992). Various amounts of the venom dissolved in 100 µl of PBS (pH 7.2) were 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 s. Plasma incubated with PBS alone served as control. In the neutralization assays, constant amount of venom was mixed with various dilutions of egg yolk Antibodies. The mixtures were incubated for 30 min at 37°C. Then, 0.1ml of the mixture was added to 0.3 ml of citrated plasma and the clotting times recorded. In the control tubes, plasma was incubated with either venom or IgY, alone. Neutralization was expressed as the effective dose (ED), defined as the ratio of ml antivenom (IgY)/mg venom at which the clotting time increased three times when compared to that of plasma incubated with double MCD of the venom alone. Statistical evaluation was performed using the XLstat 2007 and SPSS 10 softwares. $P< 0.005$ was considered statistically significant.

Egg-laying hens were immunized with the *E. carinatus* venom and the antibodies were purified from egg yolk by the four different purification methods. These methods were compared in terms of total protein content, immunospecific anti *E. carrinatus* activity *in vitro* and *in vivo* neutralizing capacity of IgY against *E. carinatus* venom. The total IgY concentrations varied from 1.6 to 7.0 mg per ml of the egg yolk. The IgY preparations purified by the PEG and ammonium sulphate precipitation (PEG-AS) method contained significantly more total protein as well as IgY than did those purified by the other three methods. The proportion of IgY of the total protein purified by the chloroform method, was approximately 80% as compared with more than 90% IgY purified by PEG-AS precipitation (Fig. 1).

Indirect antigen capture assay (IACA) showed that the antibodies were generated in chicken against the injected *E. carinatus* venom antigens. A high peak titer of more than 1:10000 monovalent antibodies was observed in immunized chicken egg yolk (Fig. 2). The immunospecific anti *E. carinatus* activity was higher in the IgY preparations obtained by the method based on polyethylene glycol (PEG) and ammonium sulphate precipitation method when compared with the activity of the IgY preparations purified by the three other methods. The concentrations of total protein and IgY purified by polyethylene glycol (PEG) and ammonium sulphate precipitation method were significantly higher than those in corresponding preparations purified by the other methods.

The antivenom potential of chicken egg yolk antibodies extracted by the different purification methods were tested against the *E. carinatus* venom by *in vivo* and *in vitro* methods. The LD_{50} of the venom was

Table 1. *In vivo* and *in vitro* neutralization tests for testing antivenom potential of chicken egg yolk antibodies against the *E. carinatus* venom.

Purification Methods	*In vivo* and *in vitro* neutralization tests			
	Lethality (PD50)	Haemorrhagic activity	PLA_2 activity	Procoagulant activity
Water dilution	1.33	1.29	1.25	1.22
Chloroform extraction	1.28	1.23	1.20	1.19
PEG-AS precipitation	1.25	1.21	1.18	1.17
Lithium sulphate	1.30	1.24	1.22	1.19

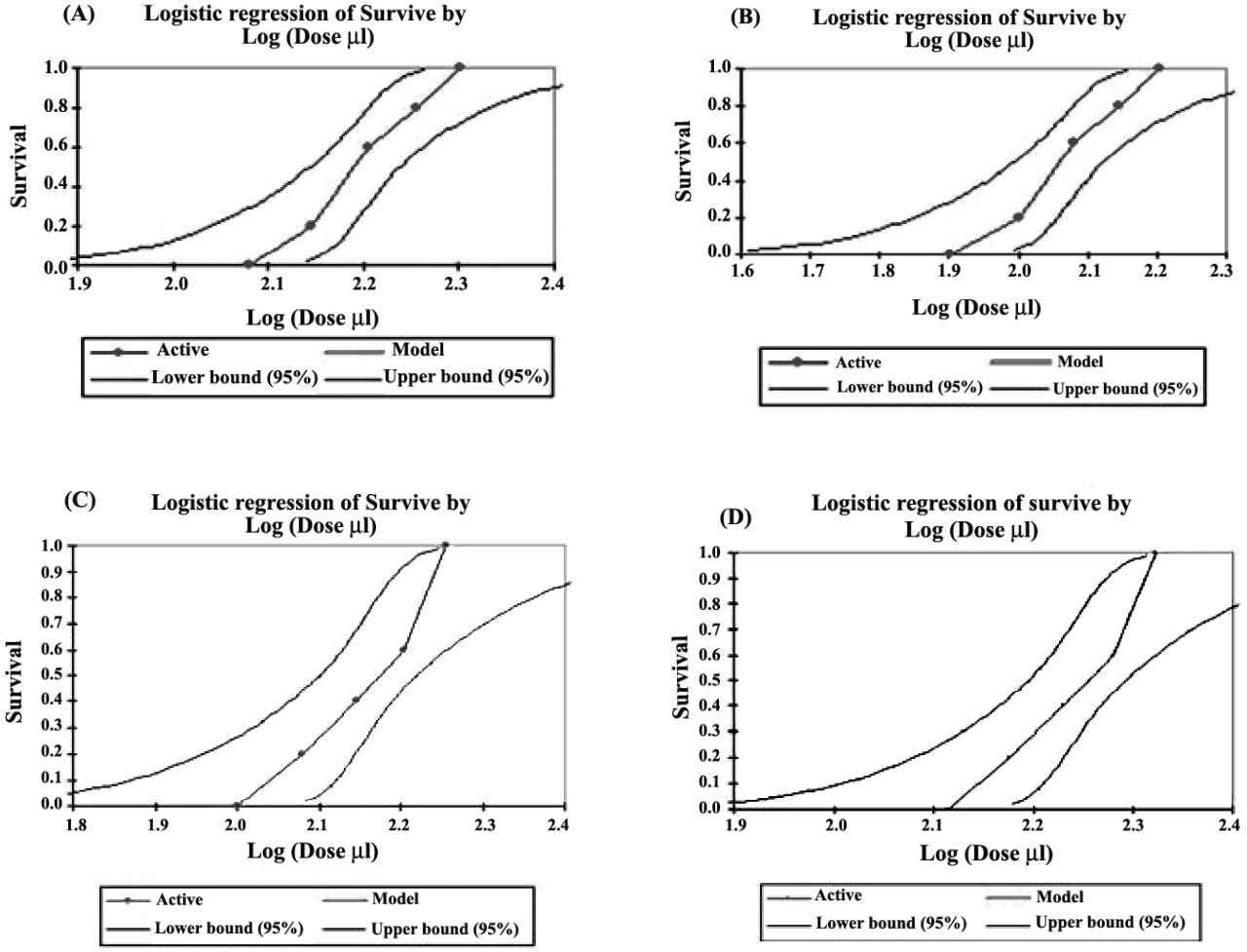

Figure 3. Neutralization of Lethality by IgY against *Echis carinatus* venom (dose-response curve). Dose-response curve for neutralization of lethality (ED_{50}) by IgY obtained by four different purification methods against *Echis carinatus* venom. A: Water Dilution Method 174.1 µl (156.2 µl-195.3 µl) B: Chloroform Extraction Method 144.6 µl (126.2 µl-165.4 µl) C: Polyethylene Glycol and Ammonium Sulphate Precipitation Method 118.5 ml (96.8 µl-128.1 µl) and D: Lithium Sulphate Precipitation Method 157.2 µl (139.4 µl-173.1 µl), $p<0.005$.

assessed using 18 g, Balb/c strain mice. About 12 µg of the *E. carinatus* venom was found to be LD_{50} for 18g of mice. It was found that 1.25 mg of IgY polyethylene glycol (PEG) and ammonium sulphate precipitation method 1.28 mg of IgY (chloroform), 1.30 mg of IgY (lithium sulphate) and 1.33 mg of IgY (water dilution method) were able to completely neutralize the lethal activity of $2LD_{50}$ of the *E. carinatus* venom (Fig. 3, Table 1).

In the case of hemorrhagic activity, the venom produced visible hemorrhagic spots and chicken egg yolk antibodies were effectively neutralized *E. carinatus* venom induced hemorrhagic activity. With regard to phospholipase activity (PLA_2), the *E. carinatus* venom was able to produce haemolytic haloes in agarose-erythrocyte-egg yolk-gel plate Approximately 10 µg of the venom produced an 11 mm diameter haemolytic halo, which is considered to represent 1U (Unit/10 µg). Chicken egg yolk antibodies were capable of inhibiting PLA_2 dependent haemolysis of sheep Red Blood Cells induced by the *E. carinatus* venom in a dose dependent manner. Approximately 120 µg of the 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 1).

Antivenom is the specific antidote for snakebite envenomation. The commercial antivenom consists of polyclonal antibodies produced by fractionating blood from horses immunized with venom. Anti-snake

venom therapy may cause various side effects such as anaphylactic shock, pyrogen reaction and serum sickness. In recent years, immunoglobulins obtained from avian egg yolks are increasingly finding favour to replace mammalian antibodies for diagnostic and therapeutic applications. In our study we compare four different purification methods for isolation of egg yolk antibodies from chicken immunized with *Echis carinatus* venom. Various purification methods were compared in terms of total protein content, immunospecific anti *E. carrinatus* activity *in vitro* and *in vivo* neutralizing capacity of IgY against *E. carinatus* venom. The total immunoglobulin Y (IgY) concentrations varied from 1.6 to 7.0 mg per ml of egg yolk. The proportion of IgY of the total protein isolated by chloroform was only about 80% as compared with more than 90% in the IgY preparations purified by PEG and Ammonium sulphate precipitation. The immunospecific anti *E. carinatus* activity was higher in the IgY preparations obtained by the method based on PEG precipitation compared with the activity of the IgY preparations purified by the three other methods. Polyclonal antibodies against Viper venom are present in the hens egg for upto 100 days after the immunization (Mayadevi *et al*., 2002). Various pharmacological activities like lethality, hemorrhagic activity, phospholipase activity (PLA_2), procoagulant activity caused by *E. carinatus* venom were studied and the neutralization of these pharmacological effects was carried out using chicken egg yolk antibodies purified by different purification methods. The results showed that the chicken egg yolk antibodies extracted by Polyethylene glycol and ammonium suphate precipitation method show better results when compared to other three methods. The pre-incubated mixture of both affinity-purified as well as partially purified anti- *E. carinatus* IgY with 2LD50 dose of *E. carinatus* venom gave 100% protection in mice when administered subcutaneously (Paul *et al*., 2007). The present experimental results indicate that chicken egg yolk antibodies were effective in neutralizing the main toxic and enzymatic effects of *E. carinatus* venom. In conclusion, egg yolk antibodies purified by Poltethylene glycol and ammonium sulphate precipitation method show high IgY content and the *in vitro* and *in vivo* neutralizing capacity is also good. Based on the results it is confirmed Polyethylene glycol and ammonium sulphate precipitation method is the effective method for isolation of chicken egg yolk antibodies.

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