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Pharmacological Characterization of *Daboia russelii* Venom and its Neutralization by Polyvalent Chicken Antibodies

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ABSTRACT
Snake bite is considered as a socio-economic problem in tropical countries. *Daboia russelii* (Russell's viper) is responsible for 30–40% of all snakebites and the most number of life-threatening bites. Antivenom is the only treatment available. Hyperimmune sera raised from horses were shown to produce adverse effects. In this study, chicken Ig Y was generated against the venom of *D. russelii* and its neutralization studies were carried out by in-vitro methods. Antivenom antibodies were generated in chicken and purified by DEAE cellulose column and the antibody titre was estimated in serum and egg yolk by ELISA which showed the increase in titres. Ig Y was subjected to protein estimation, and SDS- PAGE was carried out to study the banding pattern. High molecular weight band 180 KD was observed. Pharmacological characterization like coagulant activity, direct haemolytic assay, indirect haemolytic assay and proteolytic activity was carried out. Minimum coagulant dose was found to be 120 µg. Neutralization studies were carried out using column purified Ig Y antibodies. Current study suggests that Ig Y antivenoms are effective in neutralising the pharmacological activities induced by venom.

Key words: *Daboia russelii*, Neutralisation, Pharmacology, In-vitro assay

INTRODUCTION

One of the major health concern in India is snakebites and an average of 46,900 deaths occur annually (Mohapatra et al. 2011). Venomous snakes are common around human habitations (Kularatne et al. 2014, Suraweera et al. 2020). The most widely distributed medically important snake which can be found in all biogeographical zone is *Daboia russelii*. It has remarkable adaptability of their presence even at altitude of 2,000 m. Studies suggest that variability of their presence leads to the variability in venom proteome (Sharma et al. 2014, Kalita et al. 2017, 2018, Pla et al. 2019). Region specific surveillance of Anti-Snake Venom was justified by several factors. For all poisonous snake bites Polyvalent ASV cannot be assumed to be uniformly effective. The concept of "Big Four" is being increasingly challenged with the recognition of other snake species occurring in localized areas of country (Kumar et al. 2011, Pillai et al. 2012). The practise that is followed is prescribing polyvalent antivenom serum administering it intravenously which neutralizes the four big snake venoms (Sharma et al. 2005). Besides, other problems associated with ASV use are the occurrence of hypersensitivity reactions, lack of evidence for optimal dosing schedule, high cost, limited availability and possibility of inappropriate use (Patil et al. 2013). Using chicken describes a simple method for purifying antivenom with 90% consistency, which showed good neutralization of venom in vitro. In eggs the antibodies were found up to 100 d after the immunization, and from immunized chicken egg yolks the antivenom purified was biologically active (Mayadevi et al. 2002). The current study explains the effectiveness of chicken IgY antibodies against *D. russelii* venom in neutralising the various pharmacological activities by in-vitro methods.

MATERIALS AND METHODS

Experimental animals - Chicken

Twenty-four weeks old egg laying chickens in good health weighing ~1 Kg was obtained from Revathy poultry farm, Puliyampattii. The birds were maintained free from specific pathogen and were fed with layer mash. Maintenance of chicken was done as per the guidelines for care and use of animals in scientific research (Indian National Academy 1992).

Source of venom

Lyophilized form of *Daboia russelii* venom was obtained from Irula Snake Catcher's Industrial Co-operative Society Limited, Vedanemmeli village,

Chennai, and was stored at 4°C. Commercially available horse antivenom were also used to check the antigenic potency of the lyophilized venom. Stock solution was prepared by dissolving 10 mg of lyophilized venom in1 ml of physiological saline. (0.154M Nacl).

Immunological characterization of *Daboia russelii* venom by Immunodiffusion

Ouchterlony's Double Diffusion Method

To determine the antigenic potency of *Daboia russelii* venom, Ouchterlony method was performed (Ouchterlony 1953) with 1.0 % Agarose gel.

Estimation of total protein in D. russelii Venom

To estimate the total protein in *Daboia russelii* venom, Lowry et al. (1951) method was performed. The values were extrapolated in graph and the protein content was obtained.

Assay of protease enzyme in D. russelii venom

Protease enzyme in *D. russelii* venom was assayed by Yang (1965). At the end the mixture was filtered and the amount of tyrosine liberated was calculated by Lowry *et al.*, method. The OD was read at 750 nm and the protease activity was estimated.

Assay of acetyl cholinesterase in D. russelii venom

Acetyl cholinesterase enzyme assay was done by the method of Elman (1961). The optical density was measured at 412nm.

Protein profile of D. russelii venom by SDS-PAGE

Protein profile of *D. russelii* venom and commercially available horse antivenom was studied on 10% polyacrylamide gel electrophoresis described by Lamelli (1970). The protein profile was visualised by both Coomassie blue staining and silver staining.

Preparation of venom antigen from the stock solution

From the stock solution (10 mg/ml) 5µl of *D. russelii* venom mixed with Freunds complete adjuvant (FCA) in the ratio of 1:1 using the technique of Hebert (1973).

Immunization

Twenty-four weeks old Leghorn chicken were injected intramuscularly with *D. russelii* venom for the development of egg yolk antibodies. Booster doses were given with increase in the plain venom dose. Test bleeding was frequently done to check the presence of antivenom antibodies in serum. The eggs were collected repeatedly and stored at 4°C.

Isolation of Immunoglobulins

The egg yolk antibodies were purified by the method of Polson et al. (1980). The pooled Ig Y fraction was subjected to dialysis and further purified by DEAE cellulose column. The protein content of the eluted fraction was estimated by the method of Lowry et al. (1951). The concentrated protein was then checked for protein estimation and analysed by SDS-PAGE according to the method of Laemmli (1970).

Titration of antibodies

The concentrated chicken egg yolk antibodies were screened for their reactivity with *D. russelii* venom by ELISA procedure Gupta et al. (1992).

Pharmacological characterization of venom and its neutralization by chicken egg yolk antibodies

Direct Haemolysis Assay

The haemolytic action of *Daboia russelii* venom was studied *in vitro* by using RBC. Briefly, 5ml of citrated blood was centrifuged for 10mts at 900 rpm. 500µl of physiological saline and 50µl of RBC mixture served as a control. 500µl of distilled water with 50µl of washed RBC was used for 100% haemolysis. 500µl of physiological saline containing 50µl of washed RBC and venom, 50µl of various concentration of *Daboia russelii* venom was used to check the venom induced haemolysis. The tubes were put in a thermostat for 1hr at 37°C and centrifuged at 2000 rpm for 20 minutes. The supernatant fluid was poured off and the optical density was measured by using spectrophotometer at a wave length of 540 nm against water. The calculation of haemolysis was done by the formula

Experimental sample – Control sample
x 100/100% haemolysis

In neutralization studies 500µl of physiological saline mixed with 50 µl of RBC, 50 µl of venom, with various concentration of Ig Y and the OD was taken at 540 nm.

Indirect Haemolysis Assay (PLA2 activity)

Phospholipase activity was measured using an indirect haemolytic assay (Guiterrez et al. 1998). In Neutralisation studies the mixtures of constant amount of venom were incubated with different amount of antivenom (IgY), in microliters, for 30 minutes at 37! and 10 µl of each venom mixture was added to the wells in agarose-erythrocyte-egg yolk gels .The neutralization ability of Ig Y was compared

with that of horse antivenom.

Coagulant activity

The procedure described by Theakston and Reid (1983) and Gene et al. (1989) was followed to determine the coagulant activity. The minimum coagulant dose (MCD) was determined as the venom concentration inducing clotting of plasma in 60 seconds. Plasma incubated with PBS served as controls.

In neutralisation studies One MCD of venom was incubated with various dilutions of column purified IGY antibodies in micro litres for 30 minutes at 37! and 10 µl of each venom mixture was added to 0.1ml of citrated plasma and their clotting times where recorded. Similarly, horse antivenom was diluted and observed for the formation of clot. The neutralization ability of IgY was compared with that of horse antivenom. In control tubes plasma was incubated with PBS alone.

Proteolytic activity

Skim milked agar plates (1%) was prepared. Agar wells (2 wells per plate) were cut and 20 ìl of *D. russelii* venom was added to the plate and incubated for 24h at 37ºC. 20 ìl of PBS alone served as a control. Zone of hydrolysis of casein on milk agar plate was measured.

For neutralization assay various concentrations of egg yolk antibodies were pre-incubated with same concentration of venom for 1 hour of 37°C and these pre incubated samples were added to skim milk agar plates and incubated for 24h at 37ºC. Zone of hydrolysis of casein on milk agar plates was measured. Neutralization expressed as the ratio, mg egg yolk antibody/mg venom able to reduce by 50% the diameter of the zone of hydrolysis when compared to the effect induced by venom alone was measured.

RESULTS

Immunological Characterization of venom

Antigenic potency of venom by Ouchterlony's double diffusion technique (Ouchterlony 1953)

A high amount of reactivity was observed against *Daboia russelii* venom. Precipitation arcs were observed as a result of interaction between venom and antivenom which shows the antigenic potency of the venom (Fig. 1).

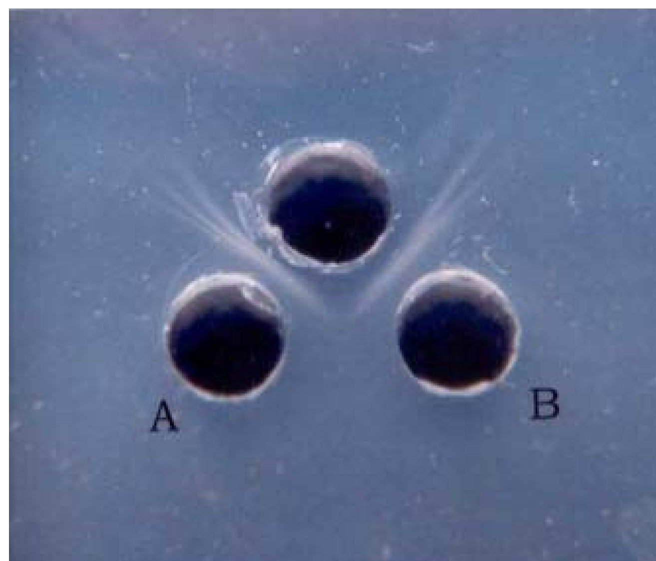

Figure 1. Ouchterlony's double diffusion technique. Central well: Polyvalent horse antivenom (A- 1:100 dilution, B-1:1000 dilution

Estimation of total protein in venom (Lowry et al. 1951)

The lyophilized *D. russselli* venom contains 70 % protein.

Protease enzyme assay in D. russelii venom (Yank 1965)

The protease activity was found to be 10.12 µg of tyrosine liberated/ml/min.

Assay of acetyl cholinesterase in D. russelii venom (Elman 1961)

Viper venoms do not have this activity. Therefore, acetyl choline esterase was nil in *D. russelii* venom.

Protein profile of D. russelii venom by coomassie brilliant blue and silver staining

The protein profile was visualised by both Coomassie blue staining and Silver staining. Different banding patterns were observed in both staining methods (Fig. 2).

Generation of antivenom antibodies in Chicken

The anti *D. russelii* antibodies were obtained through chicken serum and their egg yolk. The Ig Y antibodies were obtained by purification of chicken egg yolk using the method of Polson et al. (1980). Antibodies were observed in both serum and egg yolk.

Estimation of antibody titre by ELISA

The quantitative estimation of serum and egg yolk antibodies against *D. russelii* venom was carried out by ELISA. The results are shown in Figure 3. The

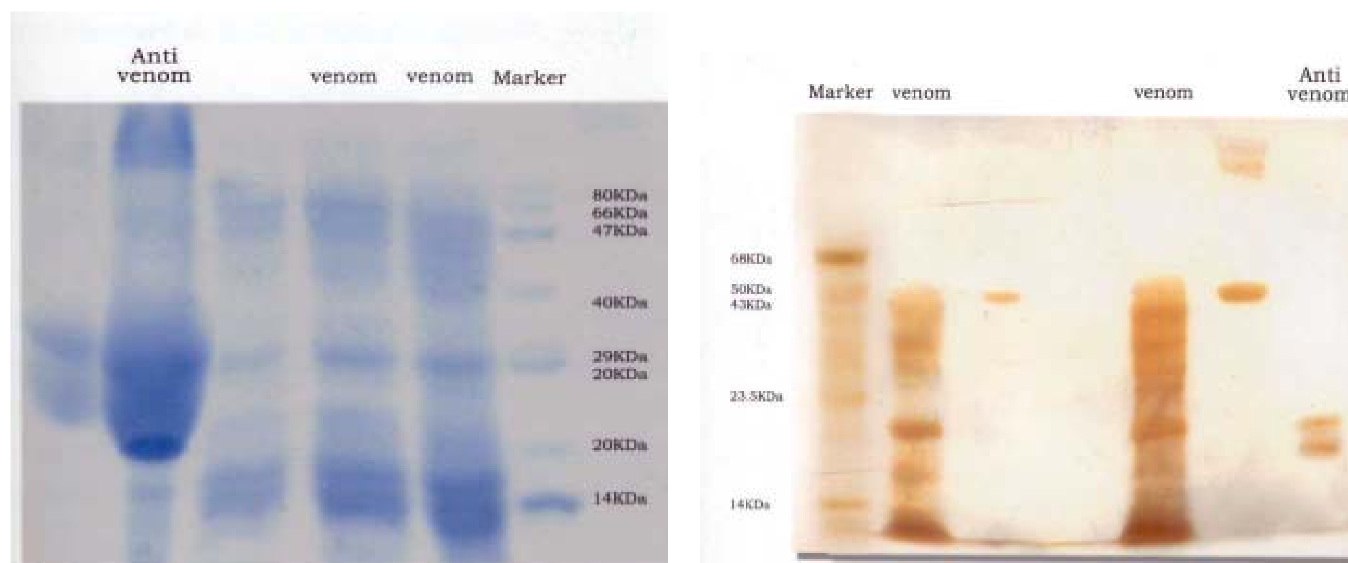

Figure 2. Banding patterns of *Daboia russelli* by Coomassie Brilliant blue and Silver staining

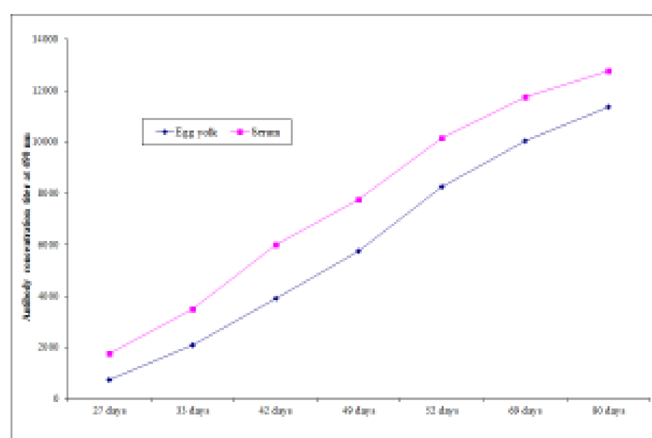

Figure 3. Estimation of antibody titer by ELISA for chicken serum and egg yolk

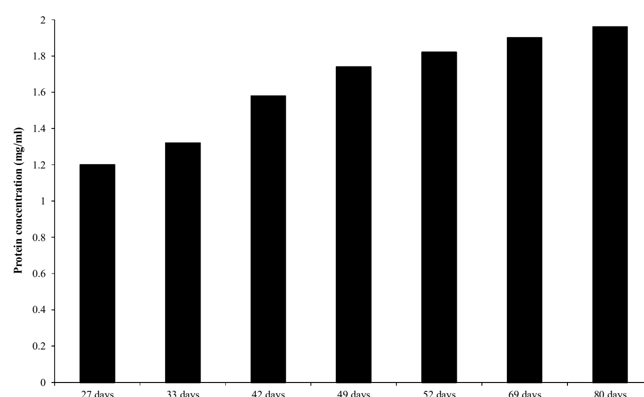

Figure 4. Estimation of protein concentration in Immunized chicken egg yolk against *Daboia russelii* venom

titre values significantly increased after immunization with *D. russelii* venom. A maximum peak was observed in 1:10,000 dilution on 80th day (Fig. 3).

Protein estimation

Protein content of the eluted IgY antibodies ere estimated by the method described by Lowry *et al.* (1951) using folin and ciocalteau reagent. Increased amount of protein content was observed (Fig. 4).

Protein profile of chicken egg yolk antibodies

Protein profile of Ig Y fraction was analysed by SDS-PAGE, according to the method of Laemmli (1970). The high molecular weight band (180KD) shows the purity of IgY (Fig. 5).

Pharmacological characterization of *D. russelii* venom and its neutralisation by chicken egg yolk antibodies

Direct haemolytic assay

Neutralization effects of IgY was tested against *D. russelii* venom by in vitro method. Direct haemolytic assay of *D. russelii* venom produced 90% of haemolysis and Chicken egg yolk antibodies showed reduction in haemolysis of RBC produced by *D. russelii* up to 20%.

Phospholipase activity (Indirect haemolytic assay)

In phospholipase activity25 µg of *D. russelii* venom produced haemolytic halo of 11 mm diameter which corresponds to one unit (Fig. 6). Chicken egg yolk

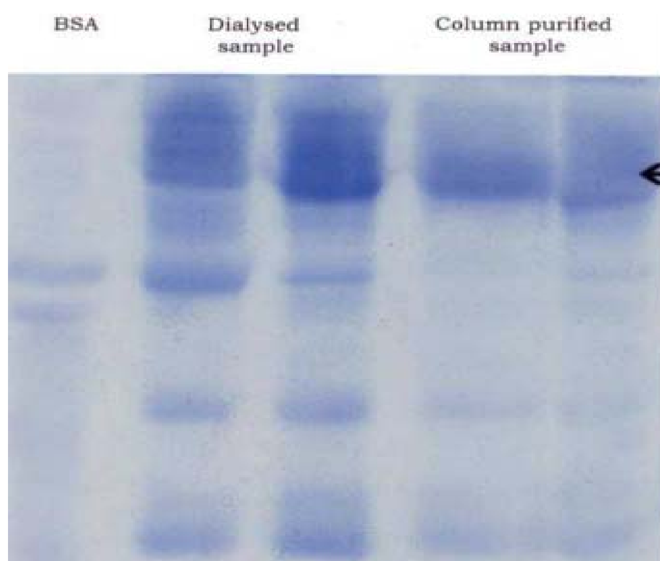

Figure 5. Protein profile of chicken egg yolk antibodies

antibodies are capable of neutralising *Daboia v*enom induced haemolysis up to 50 %.

Coagulant activity

Various amounts of venom dissolved in 0.1 ml 0f PBS were added to 0.1 ml of human citrated plasma at 37°C. Minimum coagulant dose (MCD) dose was determined as venom concentration induing clotting of plasma in 60 seconds. Controls included plasma incubated with PBS. In coagulant activity *120* µg of *D. russelii* venom able to clot human citrated plasma in 60 seconds. The chicken egg yolk antibodies effectively neutralised the venom induced coagulant activity (Table 1).

Table 1. Coagulant activity of *Daboia russelii* venom (-, No coagulation; +, Coagulation)

Concentration of venom in µg	Coagulation in 60 seconds
40	-
50	-
70	-
100	-
120	+
150	+

DISCUSSION

Snake bite results in over 137,000 annual deaths and nearly three times as many morbidities and 5.4 million people suffer from snake bite Globally (Worid Health Organisation press 2019). The majority of fatal envenomings and cases of long-term morbidity is caused by *Daboia russelii* (Suraweera et al. 2020). In the present study, snake venom *D. russelii* was checked for antigenic potency against commercially available horse antisera by Ouchterlony's double diffusion test. High amount of reactivity was observed. Total protein in *D. russelii* venom was determined by the method of Lowry et al. (1951) and was found to be 70 %. *D. russelii* venom contains proteins with different toxic properties. Assay of various enzymes present in venom like protease and acetyl choline esterase was carried out and the protease activity was found to be 10.12 µg of tyrosine liberated /ml/min. Snake venom compositions and potencies are influenced by biotic and abiotic factors. Antivenom therapy remains the only specific treatment for snake bite. A wide range of pharmacological effects in bite victims, including cytotoxicity, neurotoxicity, myotoxicity and perturbation of haemostasis was exerted by Snake venom phospholipases (Stabeli et al. 2006). Using IgY for passive immunization is gaining much importance as an alternative approach because it has more advantages like convenience, high yield and cost effectiveness than mammalian Ig G. Chicken Ig Y on oral administration was found to be effective against a variety of intestinal pathogens (Xu et al. 2011). Laying hens immunised with *D. russelii* snake venom produce antivenom antibodies that can be collected and purified from egg yolks. The

Figure 6. Phospholipase activity

antibodies first appeared two weeks after priming the hens with venoms in Freund complete adjuvant and titre increased after the administration of booster doses.

Protein profile of dialysed and column purified Ig Y fraction was analysed by SDS-PAGE by coomassie staining. The various banding patterns of these proteins were analysed. The high molecular weight band (180 KD) was observed. The extraction of IgY from egg yolk by Polyethylene glycol was found to be 80% (Pauly et al. 2011). Due to the stability IgY is also used in food formulation or supplements (Raj et al. 2004). The IgY is universally applied in medicine and research and is also expected to expand on a large scale and will be playing a key role in research Diagnosis and immunotherapy in the future (Wala et al. 2018).

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Conflict of Interest: The authors have no conflicts of interest

Authors' contributions: Both the authors contributed equally.

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