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Production of Russell's viper (*Daboia russelii siamensis*) antivenom in laying hens

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Traditional antivenoms raised in horses carry complement-mediated side effects, serum sickness and occasional anaphylactic shock. In order to circumvent these side effects and to supplement antivenom production, it was attempted to induce Russell's viper (*Doboia russelii siamensis*) antivenom in laying hens. Three injections of Russell's viper venom, a total of (500µg per hen) given at 4 week intervals yielded 1.85 gm specific chicken immunoglobulin IgY per month which is equivalent to total IgG obtained from 8 rabbits or two goats per month. The IgY antibody extracted from egg yolk with polyethylene glycol 6000 could withstand $2LD_{50}$ dose of the immunizing venom (ED_{50} =29.7µl/mouse). Antibody IgY level in egg yolk was determined by indirect enzyme immunoassay method and specificity of the antibody was checked by immunodiffusion. The antibody could be detected as early as 2 weeks after the first immunization and peak antibody levels were maintained up to 20 weeks before declining to a low level. The major advantages of the avian antivenom are the eggs from immunized hens provide a continual source of antibody, it is inexpensive to keep hens as laboratory animals and require only 3 injections using minute amounts of the venom (500µg/hen). The study highlighted that antivenom of interest could be raised by using this simple technique and the avian Russell's viper antivenom could be used for treating Russell's viper bite cases as well as to supplement antivenom production of the country.

INTRODUCTION

Russell's viper bite is endemic in Myanmar and the trend of snakebite in the country is on an increase based on hospital returns collected by the Department of Health Planning. The average annual poisonous snakebite of the whole country (1998-2005) are 8107 (6529-9600) with a case fatality rate of 7.43% (4.93-8.82%) [1]. In order to meet the requirement of increased demand of Russell's viper anti-venom, ways and means of production or supplementing antivenom production are sought for. The mainstay of management of snakebite is administration of specific antidote (antivenom) which is usually raised in horses. Administration of equine antivenom carries clinical side effects such as anticomplementary reactions, serum sickness and sometimes anaphylactic shock. In order to circumvent these side effects, production of antivenom in laying hens was attempted. The yolk of eggs laid by immunized hens has been recognized as an excellent source of polyclonal antibody [2, 3, 4]. Avian antivenoms raised against a variety of venoms have been reported [5,6,7].

MATERIALS AND METHODS

Immunisation

Four 16-week-old ISA Brown (local name CP Brown) hens were each immunized with 100µg of Russell's viper (*Daboia russelii siamensis*) venom (Myanmar Pharmaceutical Factory, MPF) in complete Freund's

adjuvant (CFA) (Gibco) on day 0 subcutaneously at six sites (both sides of the abdomen, both breasts and in both wings). The hens were further immunized with 200 μg of the same venom in incomplete Freund's adjuvant (IFA) (Gibco) on days 28 and 56.

This study was carried out in the Lane gone village of Shwepyithar Township, Yangon division from March to August 2006 which lasted for 5 ½ months. Daily collection of eggs began 2 weeks after the first immunization until the end of the study. Eggs were labeled and kept in a sand pot embedded in the ground with daily watering of the sand before being dispatched to the Department of Medical Research (LM) every weekend. They were stored at 4°C until analysed.

Collection and purification of antivenom

Chicken immunoglobulin IgY (IgG) was extracted according to the method of Polson *et al*. [8]. The broken yolks were blended with 7 volumes of egg extraction buffer (0.01 M PBS, pH 7.5) to improve antibody yield and polyethylene glycol 6000 (PEG) was added to a concentration of 3.5%. The mixture was incubated for 30 minutes with stirring and then centrifuged at 10,000 rpm for 30 minutes. After centrifugation, the precipitate was discarded. The supernatant was decanted, filtered through gauze to remove the lipid layer and PEG was added to it to a final concentration of 12% and stirred for 30 minutes, followed by 30min of centrifugation at 10,000 rpm. The supernatant was then discarded and the pellet dissolved in 0.01 M PBS, pH 7.5 and centrifuged twice to remove the PEG. The crude IgY pellet was dissolved in the original volume of egg extraction buffer 0.1 M PBS pH 7.5 and stored at 4°C until analysed.

Characterisation of immunogen (venom)

Determination of Median Lethal Dose

The lethal toxicity of the Russell's viper venom (*Daboia russelii siamensis*) was assessed by intravenous injection of 0.2 ml of the venom in physiological saline into tail vein of 18-20 gm male ICR (Institute of Cancer Research) mice. Six mice were used for each venom dose. For control, six mice were injected with normal saline. Death of the animals following 24 hours after injection was noted. The LD_{50} (intravenous) of the venom was calculated by probit analysis [9].

*Determination of venom neutralising efficacy of chicken IgY antibody (antivenom***)**

Neutralisation of lethal activity of the venom by the antivenom (ED_{50}) was performed according to the WHO recommended standard test of neutralizing activity [10]. Peak purified IgY antibody samples collected at 10, 12, 18 and 20 weeks after the first injection were tested for venom neutralizing activity. Briefly 100μl of a fixed amount ($2LD_{50}$=9.6 μg) of Russell's viper venom was incubated with an equal volume of varying amount of chicken IgY antivenom for 30 minutes at 37°C and injected into ICR mice and death of the animals within 24 hours following the injections were recorded. The ED_{50} was calculated according to the recommended WHO method [10].

Immunodiffusion

Antigen-antibody reaction was performed in 1.5% agarose gel plate using Ouchterlony's double diffusion methods [11]. IgY antibody (antivenom) samples collected at 10, 12, 18 and 20 weeks after the first injection and 1:4 dilution of Russell's viper antivenom manufactured by Myanmar Pharmaceutical Factory (batch C 98011, expiry 4/2001) were put up against 1mg/ml of the immunizing Russell's viper venom in a moist chamber at room temperature for 24 hours. The plate was washed with physiological saline and stained with 0.025% w/v Coomassie brilliant blue R-250.

Determination of antibody levels in egg yolk of immunized hens

IgY antibody levels in egg yolks of the immunized hens were determined by

indirect enzyme linked immuno assay (EIA) [12]. In brief, 96-well microtitre plate (Nunc-Immuno U) was coated with 100µl of 2 µg/ml of Russell's viper venom in 0.01 M coating buffer pH 9.6 overnight at 4°C. The plate was then washed with PBS-Tween, remaining unbound free binding sites were blocked with 3% BSA-0.01 M PBS for one hour at 37˚C. After washing with PBS-Tween, 100µl of samples (chicken IgY) were added to the wells and incubated at 37˚C for one hour. The plate was washed again with PBS-Tween 20 and 100µl of 1:10,000 dilution of alkaline phosphatase conjugated rabbit anti-chicken IgY (IgG) (Sigma) was added and incubated for one hour at 37˚C. The plate was washed with PBS-Tween 20 and the final reaction revealed by adding 100 µl of substrate made up of 5 mg/ml PNP (P- Nitrophenyl Phosphate) in 10% diethanolamine buffer pH 9.8. The reaction was stopped after 30 minute incubation at room temperature by adding 50µl/well of 3M NaOH to the wells. The colour developed was read at 405 nm wavelength in EIA reader (Humareader).

Determination of the IgY concentration and yield per ml of yolk.

The absorbance of 1:20 diluted purified IgY in 0.01 M PBS, pH 7.5 was measured at 280 nm in 10 mm path length quartz using PBS as reference. The IgY concentration (mg of IgY per ml) was calculated by using the formula where the absorbance of 1mg/ml IgY solution at 280 nm is 1.4 [2].

Determination of specific IgY

The specific IgY concentration of the crude IgY was determined by immuno-precipitation method [2]. To 0.5 ml of the crude IgY solution, 1 ml of a 0.01% antigen (Russell's viper venom) in 0.01M PBS pH 7.5 was added and incubated overnight at 37°C. The supernatant was separated by centrifugation at 3,000 rpm for 30 min and its absorbance was measured at 280 nm. The absorbance of the crude IgY was also measured.

Determination of total protein content of IgY

The protein content of the IgY solution was determined by Lowry method [13] using a standard curve generated with bovine serum albumin (Sigma).

Polyacrylamide gel electrophoresis (PAGE)

The purity and molecular weight of the protein and its subunits were determined by performing SDS-PAGE as described by Laemmli [14] using 10% separating and 4% stacking gels. The samples were dissolved in reducing SDS buffer and heated at 95˚C for 5 min. Twenty microlitres of samples were loaded to the troughs. Electrophoresis was carried out at 200 V for 45 min. using Mini Protean ll electrophoresis (Bio-Rad). The protein bands were stained with 0.1% Commassie brilliant blue R-250 and destained with 20% methanol in 10% acetic acid until a clear background was obtained.

RESULTS

Purification of egg yolk IgY antibody

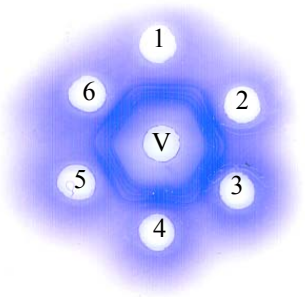
On average, laying hens (n=4) lay 22 eggs per month. The average weight of an egg is 52.38 gm ± 3.62 (1SD) (range 43.29-59 gm) and mean volume of the yolk is 13.94 ml ±1.97 (10-17.5 ml). The IgY content of the yolk is 6.05 mg/ml ±0.514 (4.82-6.87 mg/ml) or 84.38 mg IgY per egg. Purity of IgY is 87.35% (86.04-89.97%). IgY yield per month is 1.85 gm and specific IgY is 38.16% (26.12-48%). IgY protein content (Lowry method) is 6.79 mg/ml ±0.40 (5.6-6.98 mg/ml).

Immunodiffusion

The egg yolk (IgY) antivenom collected at 10, 12, 18 and 20 weeks after the first injection and the MPF antivenom showed six precipiting lines with the immunizing Russell's viper venom (Fig. 1).

SDS PAGE

SDS PAGE electrophoresis of the chicken IgY sample shows 4 major protein bands, 180 kD, 66 kD,50 kD and 31 kD (Fig. 2).



1 ~ 2 = MPF antivenom (1:4 dilution)
3 ~ 6 = peak chicken IgY samples
V = Russell's viper venom (1 mg/ml)

Fig.1. Immunodiffusion between Russell's viper venom and chicken IgY and MPF antivenoms

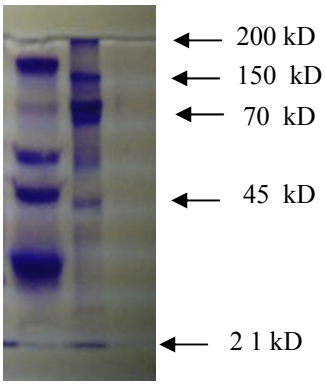


Fig. 2. SDS-PAGE electrophoresis of purified chicken IgY
Left lane = purified chicken IgY
Right lane = molecular weight markers

Development of egg yolk IgY antibodies in immunized laying hens

The IgY antibody (antivenom) was first detected in the egg yolk of the immunized hens two weeks after the first injection, reaching its peak at 2 weeks after the third immunization and maintained at its peak up to 20 weeks after the first immunization before declining to low level (Fig. 3). A transient dip in antibody level was observed at 12th week after the first immunization.

Venom neutralizing efficacy of IgY antibody (antivenom)

The $LD_{50i.v}$ of the immunizing Russell's viper venom is 4.8 µg/mouse (95% confidence levels 4.32-5.28 µg/mouse). The four peak IgY antibody (antivenom) samples

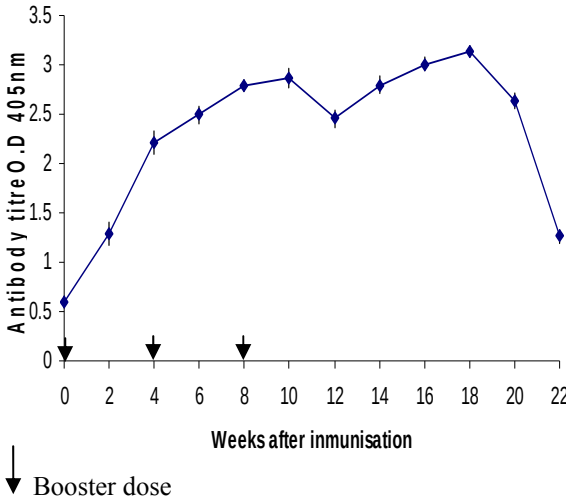


Fig. 3. Antibody titers in chicken egg yolk purified by PEG (Polyethylene Glycol 6000) after immunisation with 100 µg and 200µg of Russell's viper venom with adjuvant. The arrows indicate time of immunisation. Each point represents mean of three determinations. Bars represent standard deviations.

collected at 10,12,18 and 20 weeks after the first immunization could withstand the challenge of $2LD_{50}$ i.v. of the immunizing Russell's viper venom. The ED_{50} of the antivenoms (IgY) were 24.71 µl/mouse, 37.7 µl/mouse, 24.07 µl/mouse and 32.3 µl/mouse respectively. Mean ED_{50} was 29.69 µl/mouse (95% confidence levels 23.18-36.2 µl/mouse). One mililitre of IgY antibody (6.05 mg/ml) could neutralize 0.334 mg of the Russell's viper venom.

DISCUSSION

The study demonstrated that relatively pure Russell's viper antivenom could be obtained from the yolk of immunized laying hens. The purified IgY antibody (antivenom) could be extracted from egg yolk with polyethylene glycol. It is venom specific and has a purity of 87%. The peak IgY antibodies could withstand 2 LD_{50} of the immunizing venom. A transient drop in the antibody level at 12 weeks after the first immunization was due to ill health of the hens secondary to change in environmental housing conditions which

lasted for 4 weeks. IgY antibody could be detected in the yolk up to 100 days after three immunizations. In order to maintain peak antibody levels, subsequent boostings with the antigen are required.

In this study we used minute amount of native antigen and obtained 1.86 gm of IgY antibody per month (22 eggs/month) which is equivalent to a yield of total IgG antibody from 8 rabbits (250 gm/50 ml/month/rabbit) and that of 2 goats (1 gm/200 ml/ month/ goat) per month [15].

Earlier studies on efficacy of avian antivenoms (*Crotalus atrox* and *Trimerusurus flavoviridis*) following use of large ascending doses (mg) of the modified respective venoms and hyper immunisation schedule indicated that the antivenoms were 2 and 6.3 times more potent than the respective commercial equine antivenoms [6]. In the present study the efficacy of the IgY antivenom was found to be two times less potent than the MPF Russell's viper antivenom. It is likely that use of minute amounts of crude Russell's viper venom (500 ug per hen) with short immunization schedule (three injections) could have attributed to it. Ideally in order to have antivenom capable of neutralizing all toxic components of the venom, unmodified venom should be used rather than relying on detoxification of venom which could lead to reduction or complete loss of important antigen [16]. Formal toxoiding of Russell's viper venom resulting in reduction in biological activities of the venom has been reported [17]. For this reason, we used unmodified venom for immunization which limits use of high doses of the venom unless it is detoxified. However, the efficacy of IgY antivenom could be increased by either concentrating the final product or use of a slightly higher dose of venom or modified venom with hyper immunisation carried out in earlier cases [6].

Egg laying capacity of the laying hens declines after 100 days with slow recovery of capacity. For cost effectiveness of the study, the experiment was terminated at 100 days after the first immunization which coincides with the decline in antibody level. However it could be boosted in order to maintain the antibody levels.

The advantages of avian antivenom are (a) the eggs from immunized hens provide a continuous daily source of antivenom and avoids animal bleeding, (b) IgY antibody does not fix mammalian complement thus avoiding complement mediated side effects, (c) keeping chickens as laboratory animals is inexpensive (requires almost the same procedure as keeping other laboratory animals), (d) IgY extracted from egg yolk is pure and venom specific and (e) antibody productivity of an egg laying hen is much greater than that of a similar sized mammal, (f) purified chicken antibodies have higher bio-activity than those raised in horses (g) it minimizes administration of large volumes of foreign protein to the patients and (h) it is safer and more economical to produce compared to horse antivenom. The use of chickens for production of antibodies is attractive from an ethical viewpoint with respect to Russell and Burch's principles of the three Rs – the principle of replacing, reducing and/or refining the use of laboratory animals when possible [18].

In conclusion the study demonstrated that pure potent IgY antibody (avian antivenom) extracted from egg yolk could be used for treating Russell's viper bite cases and also to supplement the current antivenom production of the country. Moreover, this simple technology could be used for production of important and common antivenoms and has been used as successful agents for passive and protective immunization against gastrointestinal pathogens in humans and animals [15].

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