Ciência Rural, Santa Maria, v.54:02, e20220639, 2024
http://doi.org/10.1590/0103-8478cr20220639
ISSNe 1678-4596
MICROBIOLOGY

Antivenom production in chicken against Sind krait (*Bungarus sindanus*) venom and its efficacy assessment using different immunoassays

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ABSTRACT: Present study aimed for detection, purification, quantification of Sind Krait (*Bungarus sindanus*) antivenom from chicken eggs and to determine extracted antivenom efficacy in mice. Hens' three groups were immunized by sub-lethal doses of Sind Krait venom with adjuvant paraffin oil+lecithin. Booster doses were injected subcutaneously on pectorals muscles at multiple sites after every two weeks upto eight weeks. Antibodies-IgY produced against Sind Krait venom was purified form eggs' yolk by precipitation method with PEG-6000. Purified antivenom-IgY protein contents were quantified by Nanodrop-photometer, purity accessed by SDS-PAGE, specificity checked by Ochterloneys method and titer estimated by indirect ELISA. Antivenom efficacy was assessed in albino mice. Purified antivnom-IgY exhibited single protein band 180-190 kDa on SDS-PAGE under non-reduced condition and two-bands 63 - 65 kDa and 22 - 25 kDa correspondingly under-reduced condition. Immunodiffusion exhibited sharp precipitation lines of immune-complex (venom and extracted-IgY). In all groups (G1, G2 and G3) antivenom level sharply increase from 3rd to 4th week and maintained thereafter. G2 and G3 presented high titer upto 1:2048 dilutions, while G1 showed upto 1:1024 dilutions, as tested by indirect ELISA. In neutralization assay ED_{50} dose of G2 and G3 obtained antivenom was 400.23 µg/mice for more than twofold LD_{50} dose of venom and 100% protection was at 508.84 µg/mice that completely neutralized highly lethal dose of venom. But G1 ED_{50} was 405.66 µg/mice and provides 100% protection at 554.21 µg/mice. Extracted antivenom, against Sind Krait venom were highly pure, and with high neutralization capacity were produced successfully from eggs yolk first time in Pakistan.
Key words: chicken IgY, immunization, *Sind krait*, venom, paraffin oil and lecithin adjuvant, Pakistan.

Investigação e desenvolvimento de imunoensaios comparativos para produção de imunoglobulinas contra veneno da cobra (*Bungarus sindanus*) em aves

RESUMO: O presente estudo teve como objetivo a detecção, purificação, quantificação do soro antiofídico em ovos de galinha e determinar a eficácia do soro antiofídico extraído em camundongos. Os três grupos de galinhas foram imunizados por doses subletais de veneno de cobra *Sindhi krait* com óleo de parafina adjuvante + lecitina. As doses de reforço foram injetadas por via subcutânea nos músculos do peito em vários locais após cada duas semanas até oito semanas. Anticorpos-IgY produzidos contra o veneno de Krait foram purificados da gema de ovo pelo método de precipitação com PEG-6000. Os conteúdos de proteína antiveneno-IgY purificada foram quantificados por fotômetro nanodrop enquanto a pureza foi acessada por SDS-PAGE. A especificidade do antiveneno-IgY foi verificada pelo método de Ochterloneys e o título foi estimado por ELISA indireto. A eficácia do antiveneno foi avaliada em camundongos albinos. A IgY purificada exibiu uma única banda de proteína 180-190 KDa em SDS-PAGE sob condição não reduzida e duas bandas 63 - 65 KDa e 22 - 25 KDa correspondentemente condição sub-reduzida. A imunodifusão exibiu linhas de precipitação nítidas de imunocomplexo (veneno e IgY extraída). Em todos os grupos (G1, G2 e G3) o nível do soro aumentou acentuadamente da 3ª para a 4ª semana e manteve-se a partir daí. G2 e G3 apresentaram títulos elevados até diluições de 1:2048, enquanto G1 apresentou diluições de até 1:1024, testado por ELISA indireto. No ensaio de neutralização, a dose ED50 de antiveneno G2 e G3 obtida foi de 400,23ug/camundongos para mais de duas vezes a LD50 e 100% de proteção foi @ 508,84ug/camundongos que neutralizam completamente a dose altamente letal de veneno. Mas G1 ED50 foi de 405,66ug/camundongos e forneceu 100% de proteção @ 554,21ug/camundongos. Antivenenos extraídos, contra veneno de *Bungarus sindanus* eram altamente puros, seu título de anticorpos e capacidade de neutralização foram produzidos com sucesso a partir de gemas de ovos de galinhas imunizadas pela primeira vez no Paquistão.
Palavras-chave: IgY de galinha, imunização, Paquistão, *Sindi krait,* veneno, óleo de parafina e adjuvante de lecitina.

INTRODUCTION

About five million cases of snake-bite occurs and cause about 100,000 deaths annually, worldwide (BRUNDA et al., 2006). In Pakistan forty-thousand snakebite cases and about twenty thousand deaths are recorded annually (IQBAL et al., 2014). Due to deficiency of anti-venoms, annually millions of snakebites making it a grave health issue (CHENG & WINKEL, 2003). Kraits (genus *Bungarus*) belongs to family Elapidae are venomous and active at night. In Pakistan Kraits are found in coastal lowlands, east and northward to Waziristan and Quetta regions. Sind Krait (*Bungarus sindanus*) reported from Bahawalpur, western Tharpakar & Bhawalnagar. Based upon mice LD50 dose some species of *Bungarus* are among the deadliest snakes existing on land in the world (KHAN, 2002; WHITAKER, 2004).

The neutralizing agents against snake bite usually are developed by injecting horses with booster

Received 11.18.22 Approved 05.02.23 Returned by the author 06.19.23
CR-2022-0639.R2
Editor: Rudi Weiblen

doses of venom to bring about top value neutralizer titers (ESTRADA et al., 1992). A range of secondary distresses exists by the application of these anti-venom, for instance serum sickness, hypersensitivity (SCHELLEKENS, 2008) and failure of kidney which can be reduced by using fundamentally pure antivenom (DEVI et al., 2002). In order to abolish unpleasant influences of antivenom, it is required to accomplish antivenom in equitably purified form. For this purpose birds are suitable and proficient sources of antibodies (IgY) (SELLS et al., 2001; SCHADE et al., 2005).

Birds are mostly preferred for the reason of their phylogenetic distance from the mammals (IGOR & WERNER, 2010). Ample quantities of antibodies (IgY) are transferred to the yolk of eggs that reduces the offensive procedures of blood sampling. Preparation of effective, secure and inexpensive antibodies is greatly important. Antivnom (IgY) preparation from small quantities of venom is required for immunization, the ease in eggs collection and simple purification procedures to improve the proficiency of immunological assays, makes IgY uses attractive and convenient. Imperative matter of concern for Pakistan is to produce low-cost and effective antivenom domestically. Pakistan exports antivenom on a large scale from India, regardless of its efficacy against native snake species (OH et al., 2019). Indian polyvalent antivenom proved moderately effective for Pakistani *Bungarus* species, high initial doses of Indian antivenom are administered to patients as compared to locally made antivenom (QURAISHI et al., 2008). The Objectives of the study were detection, purification, quantification of anti-venoms in the yolk of chicken eggs and determination of extracted antibodies efficacy against Sind Krait venom.

MATERIALS AND METHODS

Experimental animals

Hens: Twenty Golden misri hens approximately of age 14-16 weeks and of body weight ~1 kg at egg laying stage (laying 6-7 eggs/week each), bought from local poultry farm were kept in standard size cages at animal house of Zoology Division, BZU (Bahauddin Zakariya University) Multan. Primarily for a period of 10 days, the hens were acclimatized to ambient environment. Two groups of hens were established as, control group (5 hens) and experimental groups (15 hens). Experimental hens were further divided in to three subgroups as, group 1 , group 2 and group 3 named as G1, G2 and G3 respectively, 5 hens each, for different immunization doses of Sind Krait venom. The hens were given feed (No. 13 feed for laying hen) a conventional formulated feed" and water *ad libitum*.

Mice: As model animal Albino mice were used, to check potency of prepared antivenom, to calculate LD_{50} and ED_{50} dose rates. Albino mice were gotten from Department of Pharmacy, BZU Multan. Animal handling part of experiment was maintained at animal house of Zoology division, BZU, Multan. Light, temperature and aeration was maintained constantly throughout experimental period. The animal research trial was conducted according to the ethical committee guidelines as per Bahauddin Zakariya university rules.

Venom: Sind krait (*Bangarus sindanus*) venom was collected from inhabitants of the Cholistan Desert (with the assistance of native traditional experts), that was used for immunization of birds. Experiment was lasts for a period of 56-60 days.

Dose preparation for immunization

Hens were inoculated with blends of Sind Krait (*Bangarus sindanus*) venom and adjuvant, for this venom dose was diluted with normal saline and diluted venom and adjuvant was mixed at 1:1 ratio. Water in oil emulsion of venom and adjuvant paraffin oil 80% + lecithin 20% (from egg yolk) was prepared by connecting three syringes with T-connector. Three syringes were connected, one syringe with diluted venom, 2nd with paraffin oil and lecithin adjuvant and 3rd was kept empty. Dose was prepared via method of step-by-step addition method as suggested by MONCADA et al., (1993) to prepare stabled emulsion.

Immunization of hens

Laying hens of weight ~1 kg was injected subcutaneously at sternum region, pectorals muscles with Sind Krait venom. According to LD_{50} dose (YASEEN & KHAN, 2022) sub-lethal doses of Sind Krait venom, were prudently chosen for immunization followed by progressively increasing three booster doses. The 1st immunization dose (at zero day) and 1st booster dose (after 14 days) were given in combination with adjuvants (krait venom and paraffin oil + lecithin adjuvant). The 2nd booster (after 28 days) and 3rd booster (after 42 days) doses without adjuvants were injected to all experimental hens. Eggs were collected on daily basis from day zero up to eight weeks, 6-7 eggs/hen/week and stored at 4 °C till use.

IgY extraction by precipitation with PEG-6000

Antivenom (IgY) was purified from eggs of immunized hens by PEG-6000 (polyethylene

glycol-6000) precipitation in three steps following Polson method (POLSON et al., 1980) with minor modifications. In 1st step yolk was separated, two folds to yolk volume 10mM PBS (pH 7.4) was mixed with yolk and according to final volume 3.5 % PEG-6000 was added, vortexed and mixed by rolling mixing and incubated for 10 minutes. After incubation it was centrifuged at 4800 rpm/min at 4 °C for 30 minutes and two layers of sample were appeared. The supernatant was poured to a new tube through filter paper. In 2nd step, according to new volume, 8.5% PEG 6000 (in grams) was added, vortexed, mixed by rolling mixing, incubated and centrifuged as in previous step. After centrifuge it appeared white color pellets at base and with watery layer on top. Now at 3rd step pellets of step 2 were collected and supernatants was discarded. Pellets were dissolved in 10 ml of 10mM PBS (pH 7.4) with glass stirrer and vortexed till pellets were dissolved completely, 12% PEG 6000 (10ml = 1.2g; w/v) vortexed, mixed by rolling mixing, incubated and centrifuged as in 2nd step. At the end of this step final IgY pellets were obtained and dissolved in 800µl 10mM PBS by glass stirrer, vortexed to dissolve pellets completely after air bubbles disappear, final IgY was transferred to 2ml tube. Tube was rinsed again by 400µl 10mM PBS (pH 7.4) and finally 2ml of purified IgY was obtained from each egg.

Obtained IgY was dialyzed overnight through dialysis tubes (MCWO 25KDa) in 0.1% normal saline and on next morning saline was replaced by 10mM PBS (pH 7.4) for three hours. Dialyzed IgY was centrifuged via spin column (EZ10 spin column) to remove contaminants and final clear and pure IgY was stored at -20 °C.

Protein quantification of obtained IgY (antivenom)

After purification of antivenom (IgY) from eggs yolk by precipitation with PEG-6000, obtained IgY (antivenom) protein contents (µg/ml) were measured photometrically at protein A280 nm wavelength by Nano photometer by applying an extinction coefficient 1.33 for IgY (PAULY et al., 2011).

Purity of obtained antivenom (IgY)

Obtained anti-venom (IgY) purity was observed by Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) using 6% stacking gel & 12% separating gel by Laemmli method (LAEMMLI, 1970) under reduced and non-reduced conditions. To visualize protein bands on gel Coomassie Brilliant Blue R 250 (CBB R-250) was used.

Antivenom specificity by Ochterloneys immunodiffusion method

Specificity of antivenom (IgY) was detected via Ouchterlony's immunodiffusion method (OUCHTERLONY, 1949) by using 1% agarose. For Specific binding of extracted antivenom with Sind Krait venom, 20 µl venom was added in the middle well and 30 µl of extracted antivenom (IgY) in outer wells and At 4 °C after 96h, was observed for precipitation lines, where antivenom (IgY) and Sind krait venom meets, forms immunocomplex (precipitins) and white milky color lines appeared. Precipitin lines revealed specificity of antivenom (IgY) and Sind Krait venom that forms a complex.

Antivenom titer by indirect ELISA

The obtained antivenom (IgY) titer was further assessed through indirect ELISA method. Microtiter plates were coated with different concentrations of Sind Krait venom in order to calibrate microtiter plates coating with antigen to obtain best results of antigen and antivenom binding. For titer analysis, flat bottom microtiter plates were first coated with mixture of (venom + coating buffer). Krait venom (6.86 µg/well), and coating buffer 200 µl/well (carbonate and bicarbonate buffers) was applied at room temperature for 1 hour or at 4 °C overnight. Wells were washed with PBS-T 200 µl/well five times, after washing the non-binding sites were blocked by 2% non-fat dry milk (skimmed milk) and PBS-T 200 µl/well for 2 hours at room temperature. Subsequently, blocking the wells were again washed five times and antivenom antibody (IgY) was added with appropriate dilution and incubated for one hour at 37 °C. After one hour incubation, wells were washed with PBS-T 5 times and 200 µl secondary antibodies (rabbit antichicken IgY) conjugated with horse reddish peroxidase (1:1000 dilution) was added into each well and incubated for one hour at 37 °C. After incubation wells were washed 5 times and 150µl of TMB was added into each well. The reaction color started to appear and reached at peak after 30 minutes which was stopped by addition of 3M H_2SO_4 50 µl/well. Plates were read by ELISA reader at 450 nm wavelength and OD_A was attained.

Neutralization assay of anti-venom (IgY)

Sind Krait venom toxic effect neutralization was evaluated by mixing > 2 fold LD_{50} dose (challenging dose) of krait venom with obtained antivenom (IgY). Ten groups of mice (5 each) were established and kept at laboratory conditions. One control group and 9 experimental groups were used for neutralization assay. Control group was injected

mixture of venom 9.148 µg + IgY 500-100 µl (obtained from eggs of non-immunized or control hens) and experimental groups were injected mixture of venom 9.148 µg + antivenom 500-50 µl (IgY obtained from hyper-immune eggs or experimental hens eggs). All groups of mice were injected same amount of venom but different amount of obtained antivenom (IgY). The venom and antivenom mixture dose was incubated at 37 °C for 1 hour before injecting to mice. Then mixture was injected subcutaneously and mice were observed for survival and death up to 96 hours. ED_{50} dose of antivenom (IgY) was calculated which can neutralize highly lethal dose of venom. One hundred percent survival or protection dose of antivenom was calculated based upon complete ability to neutralize the lethal effect of venom. ED_{50} was calculated by *Probit Analysis* (FINNEY, 1952) with the help of computer program Minitab 17 according to WHO guidelines.

RESULTS

Immunization of hens

According to the LD_{50} dose, a sub-lethal dose of Sind Krait venom was used for immunization. 1st immunization dose was injected to all groups as G1 = 5.49, G2 = 7.32 and G3 = 10.98 µg/kg, after 1st immunization dose 2nd, 3rd and 4th booster doses to G1 (7.32, 10.98 and 21.96 µg/kg), G2 (10.98, 16.47 and 27.44 µg/kg) and G3 (16.47, 21.96 and 32.93 µg/kg) respectively were injected with increased venom doses after every two-week interval up to eight week. On daily basis eggs were collected and labeled from zero day to the end of trial and stowed at 4 °C for further analysis.

Quantification of IgY total protein contents (µg/ml)

After extraction, obtained IgY titer showed an increased trend from zero day to the end of trial. Total protein contents at week eight were G1=5.330 mg/egg, G2 = 5.630 mg/egg and G3 = 5.530 mg/egg.

Purity analysis of obtained IgY through SDS-PAGE

SDS-PAGE analysis under non-reduced conditions, showed merely one band of about 180-189 kDa of IgY (antivenom) as shown in figure 1a, while under reducing condition presented two bands, one heavy chain and one light chain of about 63-65 kDa and 22-25 kDa respectively as shown in (Figures 1b).

Antigen binding of immunoglobulin by immuno-diffusion

After 96 hours, at 4 °C precipitant lines appeared where venom and antivenom (IgY) combined and an immune-complex (precipitin) was appeared in the form of white color lines. Thickness of white lines was representing an indication of best quantity of antivenom (IgY).

After 3rd immunization all Groups showed high antivenom (IgY) titer from week 3 to week 8, till the end of trial as shown in figure 2 (a, b and c). Week wise antivenom titer showed sharp lines from week 3 to 8 (Figure 3). All groups showed high antivenom titer via. serial dilution, where venom was loaded in central well and serial dilutions by 2x factor of extracted antivenom (IgY) were loaded in peripheral wells, resultanting in clear precipitation lines observed up to 1:16x dilutions (Figure 4: b and c) whereas, in G1 precipitation lines were observed up to 1:8x dilutions (Figure 4, a).

Week wise antivenom titer through indirect ELISA

All groups showed high week wise antivenom titer and after primary immunization there was no considerable increase in antivenom titer. An increase in antibody titer was observed at Ist booster dose level, given after two weeks, continued to increase sharply during 3rd to 4th week and remained stable till the end of trial (Figure 5).

Antivenom titer by serial dilution

While performing indirect ELISA for serial dilutions of antivenom, all wells were coated with 6.86 µg/well of venom but antivenom antibody was added serially diluted using 2x dilution factor to access maximum titer. Accordingly, group 2 and group 3 showed high antivenom titer up to 1:2048 dilution, while group 1 showed up to 1:1024 dilution (Figure 6).

Anti-lethal toxin effective dose (ED50) by neutralization assay

Neuterlization assay of Sind Krait venom was performed on mice ~20g. LD_{50} dose in mice was calculated as 0.2234 µg/g. All experimental groups were injected subcutaneously same lethal dose (grater than 2 folds of LD_{50} = 9.148 µg per mice) of Sind Krait venom and varying doses of obtained antivenom IgY (500 - 50 µl respectively to each group of mice) whereas, the control group was injected subcutaneously venom and IgY of pre-immune eggs (500 - 100 µl respectively to each mice). Survival of the model organism was observed for a period of 96 hours. There was no survival in control group mice but in experimental groups survival was observed at different doses of antivenom. ED_{50} dose of group 2 and group 3 for extracted antivenom (IgY) was calculated

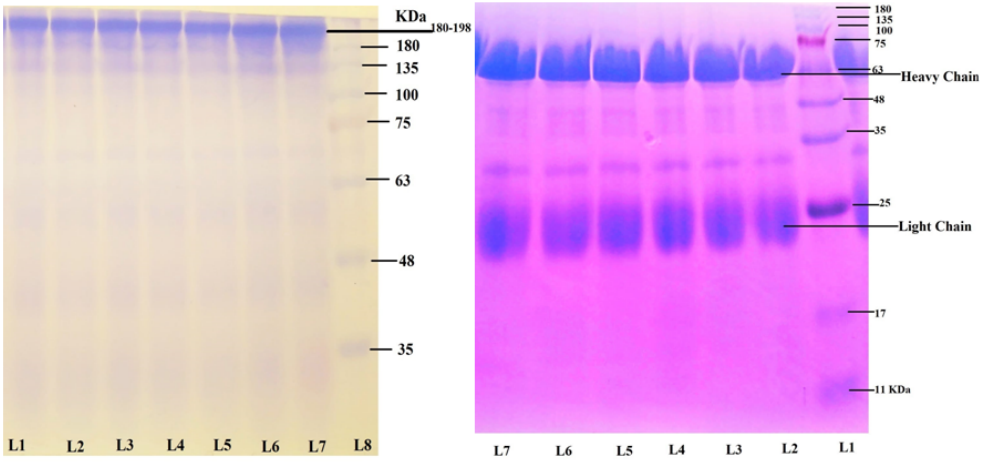


Figure 1 - (a). IgY analyzed by SDS-PAGE using 10% separating gel and 6% stacking gel under non-reduced condition (L8 showing marker with 11-180 kDa molecular weight; L1-L2, IgY group 1; L3-L4, IgY group 2; L5-L7, IgY group 3. (b). 12% separating gel and 6% stacking gel under reduced condition (L7 showing marker with 11-180 kDa molecular weight; L1-L6 showing light and heavy chain of purified IgY where L1-L2, IgY group 1; L3-L4, IgY group 2; L5-L6, IgY group 3.

as 142.177 µl/mice (400.23 µg and 393.12 µg per mice group 2 and group 3 respectively) (Table 1 and Figure 7) and showed 100% protection at the rate of 180.759 µl/mice that was 508.84 µg/mice and 499.789 µg/mice respectively. Whereas 50% protection by group 1 was provided by 152.216 µl or 405.66 µg per mice (Table 2 and Figure 8) and 100% protection at 207.96 µl/mice or 554.21 µg/mice. Apparently no signs of disturbance were observed in mice survived at high lethal dose level of venom. The model animals were healthy and active after 96 hours of survival time with no decrease in food and water intake.

DISCUSSION

In the current study, hens were immunized with an initial dose of venom/antigen (5.49 µg, 7.32 µg, and 10.98 µg, Table 1) in three different groups to check antivenom preparation response followed by booster immunizations after every two weeks as

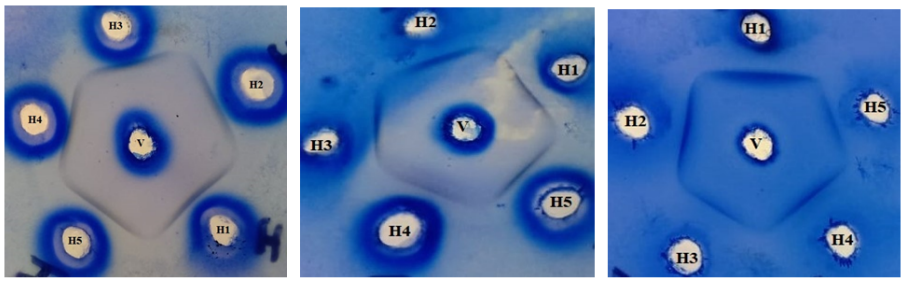
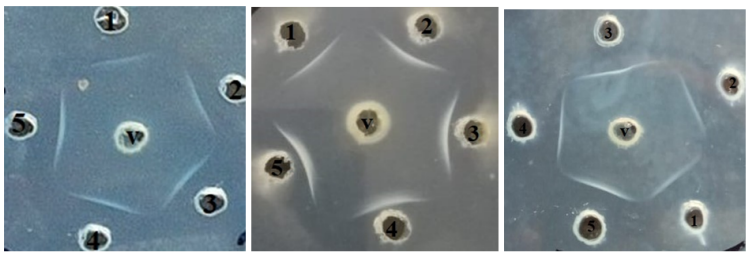


Figure 2 - (a, b and c). Immunological assessment of group 1, group 2 and group 3 purified antivenom by immune-diffusion assay against Sind Krait venom. Sind Krait venom loaded in center well and purified antivenom (IgY) loaded in outer wells. Well 1-5 shows antivenom (IgY) titer of Hen 1, Hen 2, Hen 3, Hen 4 and Hen 5 respectively at week eight after immunization.

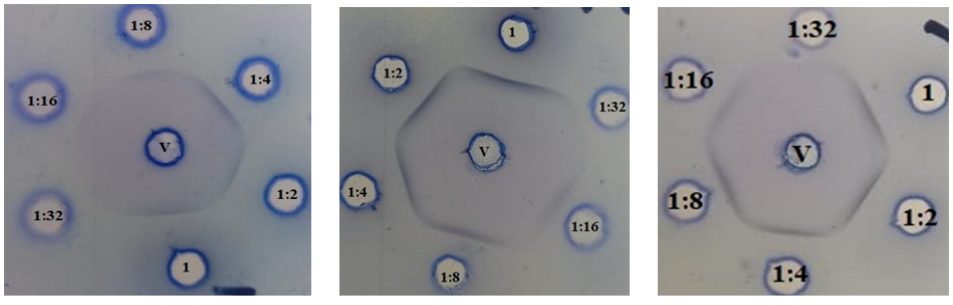


(a, G1) (b, G2) (c, G3)

Figure 3 - (a, b and c). Immunological assasment of week wise titer of group 1, group 2 and group 3 obtained antivenom (IgY) by immunodiffusion test: IgY was loaded in well 1, 2, 3, 4 and 5, purified from eggs after 3, 4, 6, 7 and 8 weeks respectively and Sind Krait venom in the central well.

by GE et al., (2020) up to a period of eight weeks as suggested by AGUILAR et al., (2014) because repeated stimulation by venom antigen B-cells can prepare antibodies than can bind antigen with greater affinity same as by WHO the use of native antigens required gradual and multiple injections to avoid the toxic effects and is required to produce efficient antibodies (WHO, 2008). In the present case, a good antivenom titer was obtained with less amount of venom when compared with usual antigens dose to immunize animals e.g. horse at commercial level (ESTRADA et al., 1992). Many side effects were reported in result to administration of horse antivenom include serum sickness and anaphylaxis shock while egg yolk antivenom was found comparatively safe (SCHELLEKENS, 2008).

Approximately 144.25 mg (IgY) was obtained from one Golden Misri hen per month at the rate of (5.770 mg/egg & 25 eggs/hen). The 2.8 mg/ml IgY (antivenom) was obtained after immunization with Sind Krait venom, is higher than IgY level measured by CARLANDER et al., (2003) as 2.21, 1.95, and 1.68 mg/mL in different genotypes (White leghorns, SLU-1392 and Rhode Island Red). Whereas, IgY was reported by BAYLAN et al. (2017) 6.658 mg/mL in the dark color group, 5.130 mg/mL in the medium color group, and 5.242 mg/mL in the light color group eggs. It has been reported that 18 immunized chickens were capable of producing the same amount of antivenom as 1 horse per year by ARAUJO et al. (2010). It was observed that total antibody (IgY) obtained from twelve eggs, was equal to IgG antibody present in 100 ml of blood. The current findings are in line with the agreement given by SCHADE et al. (2000), as one hen may be adequate to replace twelve rabbits to generate required antibodies for a duration of one year (SCHADE et al., 2000). Preparation from small amount of antigen (required for immunization), easy egg collection, continuous laying capacity of hens and uncomplicated techniques of purification of antivenom from eggs made them ideal



(a, G1) (b, G2) (c, G3)

Figure 4 - (a, b and c). Immunological assessment of group 1, group 2 and group 3 obtained antivenom titer via serial dilutions 1x-1:32x. 20 µl Sind Krait venom loaded in central well and 20 µl purified antivenom in outer wells and stained with Coomassie brilliant blue R-250.

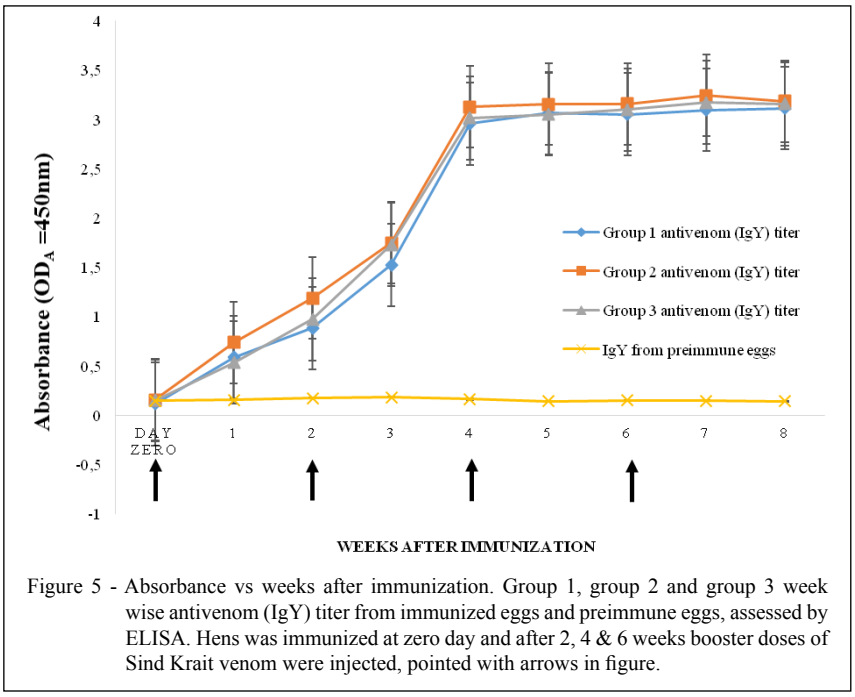


Figure 5 - Absorbance vs weeks after immunization. Group 1, group 2 and group 3 week wise antivenom (IgY) titer from immunized eggs and preimmune eggs, assessed by ELISA. Hens was immunized at zero day and after 2, 4 & 6 weeks booster doses of Sind Krait venom were injected, pointed with arrows in figure.

for antivenom (IgY) production. Antivenom started to appear in serum after one week (7-10 days) of 1st immunization with Sind Krait venom and was identified in egg yolk after two weeks progressively increased with booster doses of immunization and reached at peak after four weeks which maintained thereafter. Similar results were given by LIU et al., (2017) on antibody appearance in serum eight days after the first dose of *D. acutus* venom and in yolk at 15th day, increasing progressively along immunization procedure, and reaching a plateau after the second booster, which was afterwards maintained.

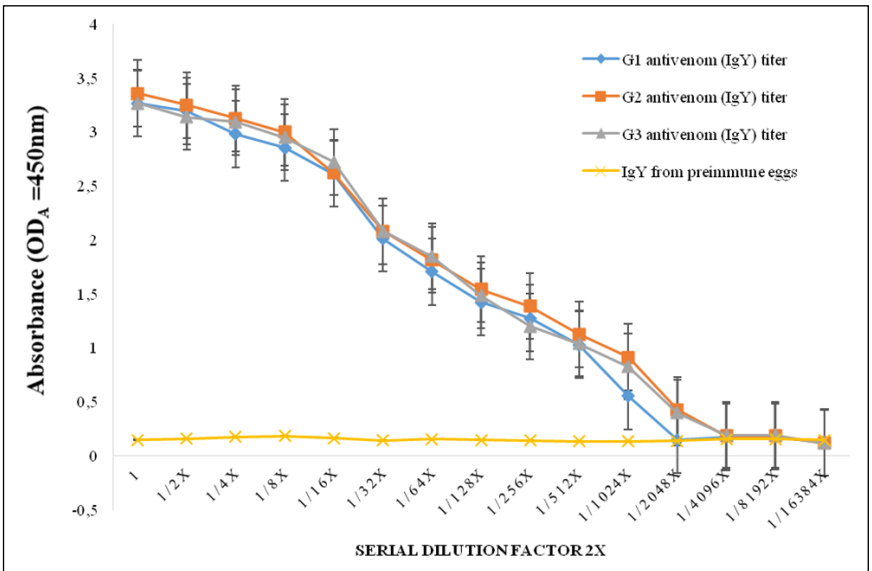


Figure 6 - Group 1, group 2 and group 3 obtained antivenom (IgY) titer by serial dilution assessed by ELISA. group 2 & group 3 showed highest titer (1:2048) and group 1 (1:1024).

Table 1 - Neutralization assay of Sind Krait venom by purified antivenom (IgY) from egg yolk of group 2 and group 3 experimental hens (able to neutralize lethal dose of venom in mice).

Control group	Experimental groups	Venom dose µg/18g mice (more than $2LD_{50}$)	Antivenom (IgY) dose in µl	No.of mice	No.of mice died	No.of mice survived
1		9.148	500-100	5	5	0
	1	9.148	500	5	0	5
	2	9.148	400	5	0	5
	3	9.148	300	5	0	5
	4	9.148	200	5	0	5
	5	9.148	175	5	0	5
	6	9.148	150	5	2	3
	7	9.148	125	5	4	1
	8	9.148	100	5	5	0
	9	9.148	50	5	5	0

The purified IgY antivenom from egg yolk, obtain by PEG-6000 method, is an inexpensive and easy method for laboratory use and we obtain increased level of protein contents after booster doses. Similar results were obtained by KUMAR et al. (2016) as IgY concentration in the egg yolk significantly increased subsequent to booster doses.

SDS-PAGE analysis of obtained IgY under non-reduced condition showed only one band near180-189 kDa as in figure 1a, whereas under reduced condition presented two bands, of one heavy chain of 63-65 kDa and one light chain of 22-25 kDa as in figure 1b. The results are in line with the findings given by LIU et al. (2010) who reported that IgY against Chinese Cobra (*Naja naja atra*) venom under reduced condition on SDS-PAGE gave one large chain of 66 kDa and one light chain of 25 kDa, separately and in non-reduced condition only one band of 182 kDa was seen. The *Echis carinatus* antivenom IgY, SDS-PAGE analysis exhibited solitary IgY band of 180 to 200 kDa molecular weight (PAUL et al., 2007). Conversely, AMRO et al. (2018) presented heavy chain with 65

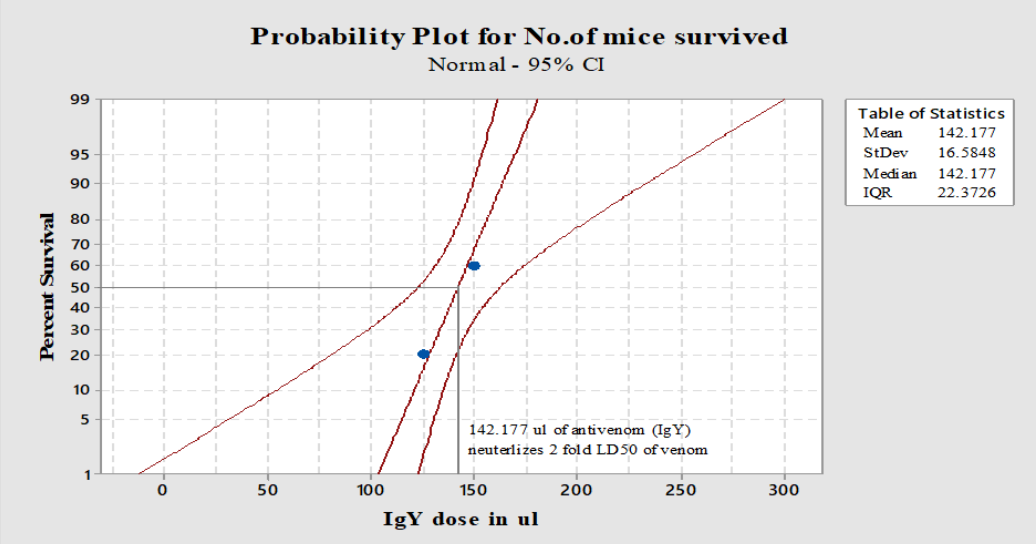


Figure 7 - Median effective dose (ED_{50}) of group 2 and group 3 obtained antivenom (IgY), able to neutralize lethal dose of Sind Krait venom in mice, calculated by *Probit analysis*.

Table 2 - Neutralization assay of Sind Krait venom final purified antivenom (IgY) of group 1 hens, able to neutralize high lethal dose of Sind Krait venom in mice.

Control group	Experimental groups	Venom dose µg/18g mice (more than $2LD_{50}$)	Antivenom (IgY) dose in µl	No.of mice	No.of mice died	No.of mice survived
1		9.148	500-100	5	5	0
	1	9.148	500	5	0	5
	2	9.148	400	5	0	5
	3	9.148	300	5	0	5
	4	9.148	200	5	0	5
	5	9.148	175	5	1	4
	6	9.148	150	5	3	2
	7	9.148	125	5	4	1
	8	9.148	100	5	5	0
	9	9.148	50	5	5	0

kDa and the light chain with 27 kDa of IgY antibodies from eggs after immunizing chicken with Zoosaloral (bacterial) cultures under reducing conditions. In another study electrophoretic investigation of Cobra and krait antivenom showed a protein of 180 kDa weight (SUNDARAM et al., 2008b).

Immuno-diffusion assessment of specific binding of venom and purified antivenom (IgY) represented very sharp lines of immune-complex (venom and antivenom) after 2 weeks from 3rd week till the end of trial (Figure 3) and at week 8 (Figure 2), though results were different with serially diluted antivenom. LIU et al. (2017) reported anti *D. acutus* venom titer by immuno-diffusion method up to 1/8x dilution whereas, in the present case immuno-diffusion results were achieved up to 1/16x dilutions (Figure 4). Thus, an indication of pure antivenom (IgY), high in titer and specific for Sind Krait venom.

The antivenom titer assessment was analyzed by indirect ELISA where all groups showed good week wise antivenom titer. After 1st booster dose there was a considerable increase in titer. Three weeks after 1st immunization and one week after 1st booster dose there was a sharp increase in titer from 3rd to 4th

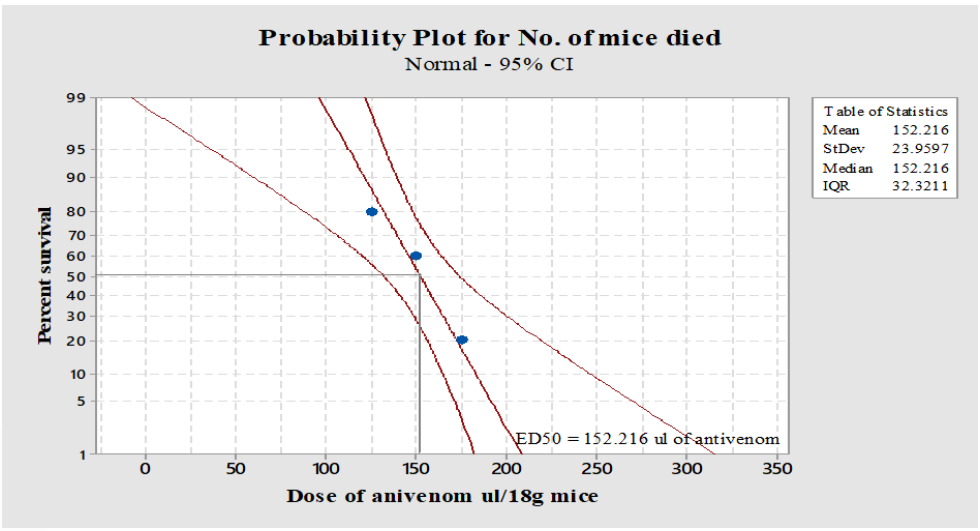


Figure 8 - Median effective dose (ED_{50}) of group 1 obtained antivenom (IgY), able to neutralize lethal dose of Sind Krait venom in mice, calculated by *Probit analysis*.

week and remained more or less stable till the end of trial (Figure 5). As reported by SUNDARAM et al. (2009) via ELISA a gradual increase in antivenom titer in egg yolk against venom coated with FCA and Bentonite reached at maximum and maintained till the end. Same response reported by ARAUJO et al. (2010). While performing indirect ELISA by serial dilutions of antivenom, all wells were coated with same amount of venom but antivenom was added serially diluted by 2x dilution factor to access titer of antivenom. Group 2 and group 3 showed high antivenom titer upto 1:2048 dilution whereas, group 1 showed slightly low antivenom titer as 1:1024 (Figure 6). ELISA titer was quite efficient with minute quanity of antivenom e.g. 0.28 µg. SUNDARAM et al. (2009) reported that hens immunized with FCA (Freund's complete adjuvant) coated venom expressed high titer at dilutions more than 1:10000 and detected minute quantity of 0.080 µg antibodies.

The neuterlization assay of Sind Krait venom was performed on model animal i.e. mice (~20g). LD_{50} dose in mice was calculated as 4.468 µg/mice or 0.2234 µg/g. Two folds of venom (greater than 2 fold LD_{50} = 9.148 µg) of Sind Krait venom and varing doses of IgY (ativenom) were tested in different model animals. In control group, venom and IgY of preimmune eggs (500-100 µl) was injected and mice were observed for 96 hours to see the survival whereas DUAN et al. (2016) determined survival up to 72 h. No survival was observed in control group mice while in experimental groups G2 and G3 ED_{50} dose of antivenom (IgY) against krait venom was 142.177 µl IgY/mice (400.23 µg and 393.12 µg per mice G2 and G3 respectively) (Table 1 and Figure 7). In group 2 and group 3, antivenom (IgY) showed 100% protection at the rate of 180.759 µl/mice (508.84 µg and 499.789 µg per mice respectively). Whereas 50% protection by group 1 was provided by 152.216 µl (405.66 µg per mice) (Table 2 and Figure 8) and 100% protection at the rate of 207.96 µl/mice (554.21 µg/mice), this slight difference may due to less venom dose was used to immunize group 1 whereas there was no difference was recorded in medium and high doses of Krait venom use to immunize hens. According to SUNDARAM et al. (2008), a median effective dose (ED_{50}) of anti-Cobra venom was reported as 4.48 mg/5LD_{50} and ED_{50} of anti-Krait venom was 3.18 mg/5LD_{50} which is very high when compared with the current experimental neutralization dose rate.

In the present research, the obtained antivenom showed 100% survival without any side effects in mice. These findings are in agreement with the results reported by DEVI et al. (2002) that egg yolk antibodies showed no mortality in mice group injected with pre-incubated LD_{50} venom while 25% mortality was recorded in same situation with commercial horse antivenom. OH et al. (2019) stated that VPAV was immune-reactive to Krait venom and moderately cross-neutralized the venom lethality with 0.25 mg/ml potency. Advanced research is needed to enhance effectiveness of specific antivenom for Sind Krait in Pakistan.

CONCLUSION

Present approach for purification of antivenom in chicken is safe and highly cost effective as compared to horses. The antibody IgY was obtained/purified from immunized chicken egg yolk. 100% survival was observed in mice with antivenom IgY after injecting Sind Krait venom without any prominent side effects. It was observed that with a small amount of venom, large quantity of antivenom can be prepared despite the effect of adjuvants used when compared with traditional method of antivenom production. Chicken are easy to handle, require less space, minimum cost in management, small amount of antigen is required to immunize hens and large number of eggs can be collected, a source of IgY. The findings depict a clear picture of IgY from chicken in greater yield of specific antivenom with minimal side effects. It is suggested that avian IgY can be efficiently utilized as an alternate to mammalian antibodies. This approach may facilitate antivenom preparation at commercial level with minimum cost. On a large scale the country is deficient in production of specific antivenom according to demand and import antivenom from India which are not fully effective and have low efficacy for local snake species present in Pakistan. It is a known fact that IgY play a cumulative role in research, diagnosis, and immunotherapy, though further research is recommended to make its applicability in humans. We conclude IgY anti-venom could be effective source to treat snake bites in animals and may be in humans but it needs to perform further assays on humans.

ACKNOWLEDGEMENTS

The authors are extremely obligated for research work facilities extended through avian ecology lab and animal house, Institute of Pure & Applied Biology, Bahauddin Zakariya University (BZU). We also acknowledge support availed from animal house, Department of Pharmacy BZU for providing albino mice for trial and University Diagnostic Lab, Faculty of Veterinary, BZU.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

Sobia Yaseen as PhD scholar conducted all the research work include animals' trial, sampling, laboratory analysis and write-up work. Aleem Ahmed Khan being supervisor conceived the idea, planned the experimental design and checked final write-up.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

All procedures on animals were performed following the guidelines approved by Ethical Committee of Bahauddin Zakariya University (BZU), Multan, Pakistan (Letter no. Biol. 4098).

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