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RESEARCH ARTICLE

Experimental antivenoms from chickens and rabbits and their comparison with commercially available equine antivenom against the venoms of *Daboia russelii* and *Echis carinatus* snakes

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ABSTRACT
In the present work, chicken (IgY) and rabbit (IgG) antibodies were generated against the venoms of *Daboia russelii and Echis carinatus* snakes and efficacy was compared with commercial antivenom. Antivenom antibodies were purified and evaluated by ELISA, SDS-PAGE, Western Blot. All antivenoms neutralized a challenge dose of $3LD_{50}$. Each ml of IgY, IgG and commercial antivenom neutralized 0.3, 0.34, and 0.6 mg of *E. carinatus* venom and 0.2, 0.22 and 0.57 mg *D. russelii* venom respectively. The present study suggests the generated antivenoms as effective in neutralizing the venoms injected, implying the scope of IgY antivenoms for further screening and evaluation.

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IgY; IgG; commercial antivenom; ELISA; western blot; neutralization

1. Introduction

Envenomation from snakebite is considered a serious health hazard in tropical and sub-tropical regions such as Africa, Asia, Latin America and Oceania affecting around 1.8–2.7 million people worldwide resulting in 81,000 to 138,000 deaths annually (Gutierrez 2017). Currently, available treatment for the envenomation caused by snake venom is the administration of antivenom produced from hyperimmune plasma of equines, which leads to undesirable side effects such as anaphylactic reactions and serum sickness (Laustsen *et al*. 2018), and if the antivenoms are well designed, they can be an effective life-saving drug. The most affected of all the regions in India, which is also the snake bite capital of the world with mortality ranging from 35,000 to 50,000 deaths annually (Choudhury *et al*. 2017). The principal common snakes responsible for causing snake envenomation in India are Indian cobra (*Naja naja*), Common Krait (*Bunagarus caeruleus*), Russell's viper (*Daboia russelii*) and Saw scaled viper (*Echis carinatus*). Snake venom is a mixture of various proteins, organic and inorganic materials. The closely related species of snakes share common proteins that vary within individual snakes depending on different environments and seasons (Pawade *et al*. 2016). The poor neutralizing ability and adverse reactions of equine antivenom have resulted in search of alternative methods for the production of antivenom. A number of research groups have produced antivenom from different animal or avian sources such as Camelid (Padula and Winkel 2016, 2017), Rabbits (Beghini *et al*. 2008, Venkatesan *et al*. 2014), Mice (Tanaka *et al*. 2016, Melo *et al*. 2017), Donkeys (Patrick Fernandez *et al*. 2009), Sheep (León *et al*. 2000), Ducks (Y-Neng Chiou 2008) and chickens (Maya *et al*. 2002, Almeida *et al*. 2008, Meenatchisundaram *et al*. 2008, Araujo *et al*. 2010, Prabhu *et al*. 2010, Duan *et al*. 2016, Navarro *et al*. 2016) so as to produce antivenom of high neutralization potential.

Identification of snakes, post snake bite envenomation depends on the information given by the patients and the conventional method of identification of venoms such as the 20 min whole blood clot test (WBCT) (Pawade *et al*. 2016, Shaikh *et al*. 2017). So far, since antivenoms have been in use for the treatment of snake envenomation, researchers are still exploring the possibilities to develop a rapid snake venom detection kit. Globally, only Australia has a Snake venom detection kit (SVDK) developed by Bio Commonwealth serum laboratories (BIO-CSL). Indian

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scenario on detection kits for snake venom remains silent though there a couple of antivenom manufacturing industries located here. Recently Pawade *et al.* (2016) and Shaikh *et al.* (2017) reported the use of Lateral flow assay (LFA) for the detection of Big four snakes of India using monovalent rabbit IgG antivenom antibodies. In the present work, a sandwich ELISA for snake venom detection have been performed using chicken IgY antivenom antibodies as well as rabbit IgG antibodies.

Chicken immunoglobulin is functionally similar to IgG from mammals. IgY is recognized as antibody of birds, reptiles and amphibians. The advantages of IgY antibodies such as lack of activation of complement system and no cross reactions with the mammalian antibodies or rheumatoid factors (Araujo *et al.* 2010, Sifi *et al.* 2018) makes IgY a possible alternative for antivenom production. As far as human trials for IgY antivenom is considered, Kiem (2000) is the only available report followed by which a number of research has been taken forward and Good Biotech, Taiwan has commercialized antivenoms with duck IgY (δ Fc) for detection as well as for therapeutic purpose for snake envenomation.

The present research work aimed to generate IgY antivenom against the venom of Saw scaled viper (*E. carinatus*) and Russell's viper (*D. russelii*) which were further characterized by ELISA, Western blot analysis and by neutralization of venoms lethal effect. To further compare the efficacy of IgY antivenom antibodies, IgG antivenom antibodies were generated in New Zealand white rabbits. These in house antivenom antibodies were compared with that of commercially available equine antivenom.

2. Materials and methods

2.1. Chemicals and reagents

Freund's complete and incomplete adjuvants, and Tween 20 were purchased from Sigma Aldrich, USA. Rabbit anti chicken IgY HRP (RAC), Goat anti Rabbit IgG HRP (GAR), Rabbit anti Horse IgG HRP (RAH), Di amino Benzidine substrate (DAB) were from Genei Laboratories, Bangalore, India. Bovine serum albumin (BSA) was purchased from Himedia, India.

2.2. Venom and LD_{50} determination

Snake venoms (*E. carinatus* – SSV, *D. russelii* – RV, *Naja naja* – Indian cobra, *Bungarus caerulus* – Common krait) were purchased from Irula snake catcher's cooperative society, Chennai, Tamil Nadu, India in lyophilized form which was reconstituted with 0.9% saline at a concentration of 1 mg/ml and was stored at −20 °C until use. The lethal activity of the venom was determined by injecting venoms (DR and EC) i.v into groups ($n = 5$) of swiss albino mice of each venom dose. The LD_{50} of the venom was calculated by the number of deaths occurring in 24 hours and was determined by probit analysis (Camey *et al.* 2002).

2.3. Commercial antivenom

Commercial Anti-snake venom (ASV) which is an equine serum manufactured by Bharat serums and vaccines was purchased from a local pharmacy store. Manufactured on 05/17 and expiry on 04/21 with batch no. A05317069. The commercial antivenom is a purified form of a F(ab)'2 portion.

2.4. Characterization of snake venoms by SDS PAGE analysis

Snake venoms (SSV and RV) were subjected to SDS PAGE. Briefly 12% gels were prepared and the venoms (20 µg) were loaded to the wells. The gel was run at 74 volts for 02:30 h. Upon complete run, the gel was washed with distilled water for 2 min and was stained with coomassie brilliant blue (CBB) (0.1%) solution (Ethanol:acetic acid:water – 25:5:70) for an hour followed by destaining in the same solution without CBB. Protein concentration was estimated by the method of Bradford (1976).

2.5. Birds and animals

21 weeks old egg laying white leghorn chickens were procured from a local poultry farm. New Zealand white rabbits weighing (2–2.5 kgs) and Swiss albino mice (18–25 g) were from PSG Institute of Medical Sciences and Research (PSG IMS and R). All procedures involving the use of animals and birds were ethically approved by the CPCSEA committee at PSG IMS and R (Approval number 385/2018/IAEC and 402/2018/IAEC).

2.6. Experimental production of IgY antivenom antibodies

Venom of SSV and RV (50 µg) were mixed with PBS and Freund's complete adjuvant. The total volume of the mixture was made up to 1 ml and chickens ($n = 2$) were immunized subcutaneously at multiple sites. The booster dose of the venom was administered with Freund's incomplete adjuvant after every 2 weeks. The

eggs were collected and stored at 4 °C. For purification of IgY antibodies, the method of Polson *et al.* 1980 was followed. Briefly, the egg yolks were separated from white, and were emulsified in PBS 1:2 ratio and were precipitated by adding 3.5% PEG and centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant was filtered and was precipitated again with 8.5% PEG and centrifuged in the same manner as mentioned above. The supernatant was discarded and the pellet was dissolved in PBS to a final volume of 10 ml and 12% PEG was suspended to the tubes and centrifuged. The resultant pellet was dissolved in 1 ml of PBS and stored in −20 °C until further use. The protein concentration of the antivenom antibodies was calculated using Bradford's method.

2.7. Experimental production of rabbit IgG

50 μg of SSV venom was mixed with PBS and Freund's complete adjuvant. One milliliter of the antigen adjuvant mixture was subcutaneously administered at multiple sites in rabbits ($n = 2$). In a similar manner, booster doses were administered. The monovalent serum was collected by cardiac puncture. IgG was purified using ammonium sulfate precipitation method. For precipitation of IgG from rabbit serum, a saturated solution of ammonium sulfate was prepared by dissolving ammonium sulfate granules in distilled water (700 g/L), resulting in 100% saturation. The prepared solution was cooled at 4 °C overnight. The serum was placed in a beaker placed on magnetic stirrer at 4 °C with an equal amount of 100% saturated ammonium sulfate solution to achieve 50% saturation. The mixture was then allowed to stand for 30 min at 4 °C and then centrifuged at 4000 rpm for 20 min. The supernatant was discarded and the pellet was washed again with 50% of ammonium sulfate solution. The pellet was finally dissolved in 0.9% saline and dialyzed against the same buffer overnight. IgG was then stored at −20 °C for further use.

2.8. Serial dilution ELISA

96 well microtiter plates were coated with the venom of SSV or RV (10 μg/ml) in carbonate buffer (pH – 9.6). The plates were allowed to stand for an hour at room temperature followed by incubating overnight at 4 °C. Plates were washed twice with wash buffer (PBS – pH -7.4 containing Tween 20 – 0.05%) and the unbound sites in the wells were blocked by the blocking solution (5% skim milk in PBS) and incubated for 3 h at RT. The antivenom antibodies (IgY, IgG and commercial antivenom) were added to the wells in increasing concentrations (in blocking buffer) and incubated for an hour at room temperature. The plates were washed thrice and the secondary antibodies conjugated with HRP (RAC, GAR and RAH) were added to the wells at a concentration of 1:1000 and incubated for an hour at RT. The plates were washed and the substrate (Tetramethyl Benzidine, TMB) was added to the wells (100 μl/well) and kept in dark for 20 min at room temperature. The reactions were stopped by the addition of 1 N sulfuric acid and the absorbances were recorded in an ELISA reader with absorbance at 450 nm.

2.9. Evaluation of purity of IgY and IgG antibodies by SDS PAGE analysis

The IgY and IgG anti SSV and anti RV antivenom antibodies were subjected to SDS PAGE analysis. Briefly 10% gels were prepared and the antivenom antibodies (20 μg) were loaded to the wells. Upon completion of the run, the gels were subjected to CBB staining and the bands were visualized.

2.10. Specificity analysis of antivenoms by Western blot

Snake venoms (SSV and RV) were run on SDS PAGE gel as mentioned (in Section 2.4). The venom samples were transferred to the nitrocellulose membrane using a semi dry blot apparatus (Hoefer 7E7OX, Thermo scientific). On successful transfer of the samples, the membranes were blocked at 4 °C overnight in 5% skim milk (prepared in TBS – pH 7.4). The membranes were washed thrice (10 min/wash) with wash buffer (TBS containing 1% Tween 20) and the primary antibodies (anti SSV and RV IgY, IgG and commercial antivenom) were added to the membranes at a dilution of 1:3000 in blocking buffer and incubated at 4 °C overnight with continuous shaking. The membranes were washed thrice with wash buffer and the secondary antibodies conjugated with HRP (Rabbit anti chicken IgY HRP, Goat anti Rabbit IgG HRP and Rabbit anti Horse IgG HRP) were added to the membranes at dilution of 1:5000 in blocking buffer and incubated at RT for 3 h. The membranes were washed and the substrate Diamino Benzidine (DAB, Genei, Bangalore) was added to the membranes and the bands were visualized.

2.11. Relative avidity ELISA

The avidity of the antivenoms (IgY, IgG and BSV antivenoms) were evaluated by using a chaotrophic ELISA. For this assay, ammonium thiocyanate (NH_4SCN) was used in different molar concentrations (0–8 M). The plates were coated with respective venoms as done for serial dilution ELISA. The plates were washed and blocked (5% skim milk in PBST) for 3 h at room temperature. The antivenoms were diluted in a single dilution of 1:100 in blocking solution and were incubated at 4 °C overnight. The plates were washed and the chaotrophic agent (ammonium thiocyanate – NH_4SCN) was added in different concentrations (0–8 M) and incubated for 15 min at room temperature. The plates were washed and the respective secondary antibodies (Goat anti rabbit IgG HRP; Rabbit anti chicken IgY HRP; Rabbit anti horse IgG HRP) diluted in the ratio of 1:1000 in blocking solution and incubated for 3 h at room temperature. The plates were washed and the substrate (TMB) was added and incubated for 20 min in dark at room temperature. The reactions were stopped by the addition of 4 N H_2SO_4 and the OD was measured at 450 nm.

2.12. Sandwich ELISA for the detection of snake venoms

96 well ELISA plates were coated with either IgY (10 μg/ml) or IgG (10 μg/ml) in carbonate buffer (pH – 9.6). The plates were allowed to stand for an hour at room temperature followed by incubating overnight at 4 °C. The plates were then washed with wash buffer (PBS – pH 7.4 containing 0.05% Tween 20). The unbound sites of the antibodies were blocked with 5% skim milk (prepared in PBS) and incubated for 3 h at room temperature. The plates were washed thrice with PBST and the respective venoms (RV, SSV) were added in different concentrations in blocking buffer (100 μl/well) and incubated for an hour at room temperature. The plates were again washed and the detection antibodies (for IgY coated plate IgG was added and for IgG coated plate IgY was added) at dilutions 1:100 in blocking buffer (100 μl/well) and incubated for an hour at room temperature. The plates were washed and the secondary antibodies conjugated to HRP (Goat anti rabbit IgG HRP; Rabbit anti chicken IgY HRP) were added at dilutions 1:1000 in blocking buffer and incubated for an hour at room temperature. After a final wash, the substrate (Tetra methyl benzidine, TMB, Sigma, St. Louis, MO) was added to each well and incubated in dark for 20 min. The reactions were stopped by the addition of 4 N $H_2SO_4$and the Optical density was measured at 450 nm using an ELISA reader.

2.13. Lethal activity neutralization

Venom samples (SSV and RV) corresponding to $3LD_{50}$ were incubated with serial dilutions of antivenom (IgY, IgG and commercial antivenom) for 30 min at 37 °C. After this, swiss albino mice ($n = 4$) were intravenously injected with the samples (0.2 ml). The effective dose (ED_{50}) was calculated with the number of deaths within 48 h post injection of the venom and antivenom mixture. All antivenoms were made in a single protein concentration of 30 mg/ml using amicon centrifugal concentrators. The neutralization potency was expressed as mg of venom/ml of antivenom and was calculated using the formula

$$P = [n - 1/ED_{50}] \times LD_{50}$$

where n represents the number of LD_{50}s used as challenge dose.

2.14. Statistical analyses

The data were statistically analyzed using ANOVA, followed by Bonferroni *post hoc* test, as required. Significance was accepted at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Data were analyzed using the statistical package Graphpad Prism (Graphpad Software 8.1, Inc, San Diego, CA)

3. Results

3.1. Characterization of snake venoms and antivenom

The venom profiling of the snake venoms was observed by SDS-PAGE analysis. The snake venoms ranged from low molecular weight proteins to high molecular weight proteins (15–100 kDa). Indirect antibody capture ELISA was performed for determining the antibody titer against the respective snake venoms. The generated monovalent IgY antivenom antibodies showed a high titer of 1:32,000 for the venom of RV whereas IgY antibodies raised against the venom of SSV showed a high titer of 1:64,000. These titer levels were obtained after 90th day post immunization. The IgG antibodies obtained from ammonium sulfate precipitation, showed good titer (1:64,000) even after 105 days post immunization (Figure 1(A,B)). The purity of the IgY and IgG antibodies were observed by SDS-PAGE analysis (Figure 2(A,B)). There were two bands observed under reducing conditions

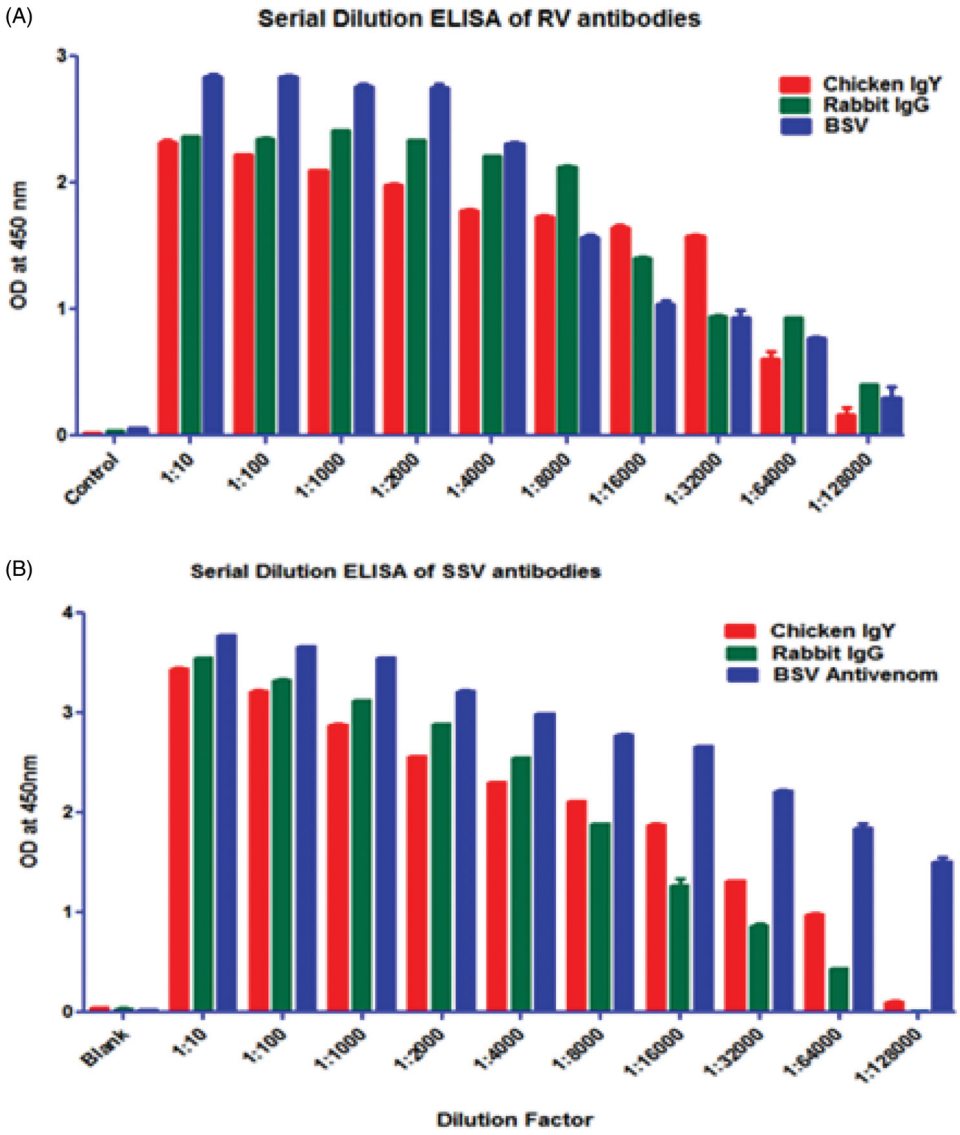

Figure 1. (A) Serial dilution ELISA (RV venom antibodies). (B) Serial dilution ELISA (SSV venom antibodies). All the samples were added in duplicates and the values are expressed in mean ± SD; significance with ANOVA Bonferroni's post-test.

for IgY and IgG antibodies respectively. The specificity of the raised monovalent antivenom antibodies and the commercially available antivenom was evaluated by western blot analysis. The generated antivenoms (IgY and IgG) against the venoms of DR and EC recognized all bands of snake venoms as shown in the SDS-PAGE of venoms (Figure 3(i,ii)).

Relative avidity ELISA was performed in order to test the venom antivenom protein binding ability. The antivenoms (IgY, IgG and commercial antivenom) were added to the wells in a single dilution (1:100) and the chaotrophic agent (Ammo. Isothiocyanate) was added to the wells in different molar concentrations (0–8 M). We observed that the IgY antivenom showed a rapid decrease (reduction in OD value) at concentration of 4 M for SSV venom, where IgY antivenom against the venom of RV was stable until 6 M concentration. The IgG antivenom and the commercial antivenom were stable until 6 M concentration (Figure 4). Sandwich ELISA was performed in order to determine the ability of the IgY and IgG antivenoms to detect the venom at lower concentrations. The IgY antivenom had a detection limit up to 0.1 ng whereas the IgG antivenom was able to detect the venom at concentration up to 100 ng (Figure 5). The antivenoms generated from chickens and rabbits were able to detect snake venoms but do not detect the species responsible for snake bite, as these antivenoms were not affinity purified and to evaluate an antibodies detection ability, Cyanogen Bromide (CnBr) activated sepharose chromatography must be performed.

Cross reactivity analysis of the generated monovalent antivenoms was evaluated by ELISA. The anti SSV

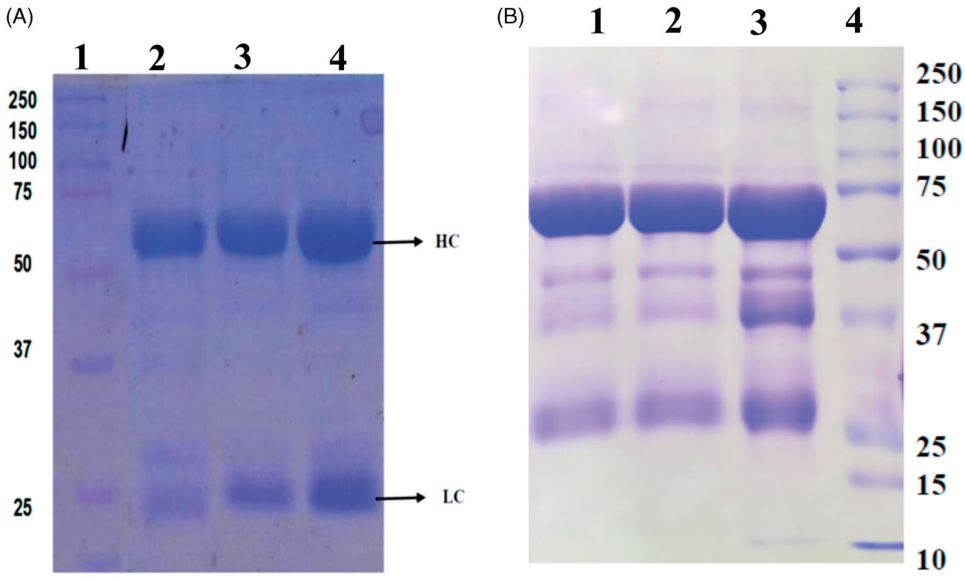

Figure 2. SDS PAGE analysis of IgY and IgG antivenom: 10% SDS gels were prepared and the samples ((A) 20 µg of IgY and (B) IgG) were run in reducing conditions at 74 volts for 02:30 h. The samples were stained using Coomassie Brilliant Blue R-250 (Ethanol:acetic acid:water – 25:5:70) for an hour and were destained in the same solution without CBB overnight. Two chains (one heavy and a light chain) at 65 and 25 kDa, respectively, were observed for IgY and 75 and 25 kDa, respectively for IgG antibodies. (A) Represents IgY antivenom antibodies, (1) Molecular marker (2) Control IgY; (3) anti SSV IgY; (4) anti RV IgY. (B) IgG antivenom antibodies (1) anti SSV IgG; (2) anti RV IgG, (3) Control IgG (4) Molecular marker.

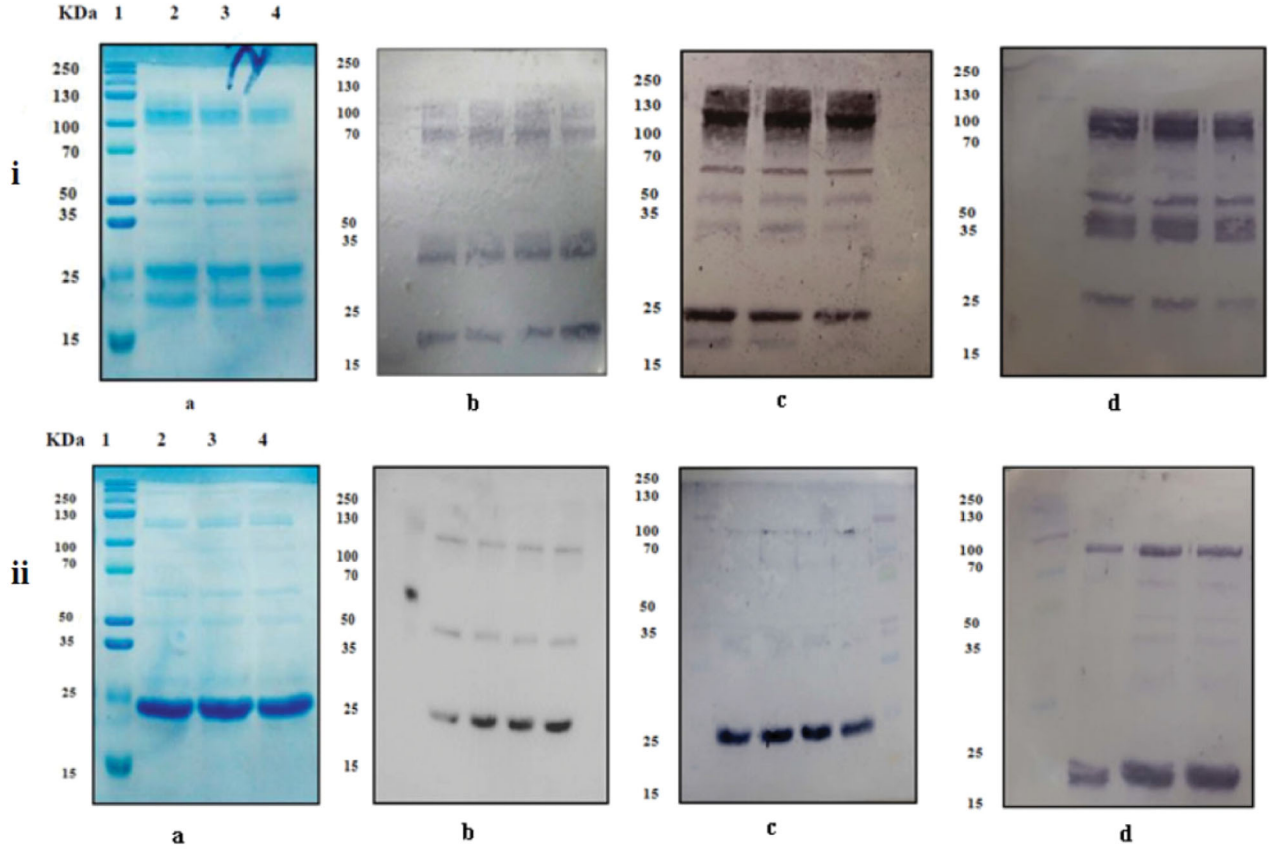

Figure 3. Western blot analyses: (i) (a) SDS PAGE analysis of SSV venom; (b) WB of anti SSV IgY antivenom; (c) anti SSV IgG antivenom; (d) commercial antivenom; (ii) (a) SDS PAGE analysis of RV venom; (b) WB of anti RV IgY antivenom; (c) anti RV IgG antivenom; (d) commercial antivenom.

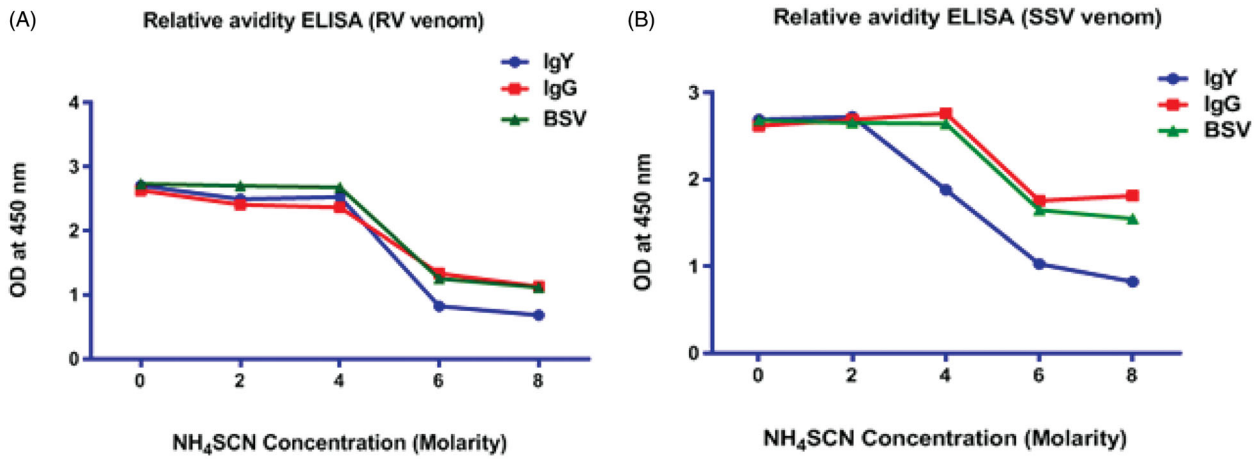

Figure 4. (A, B) Relative avidity ELISA (chaotrophic ELISA).

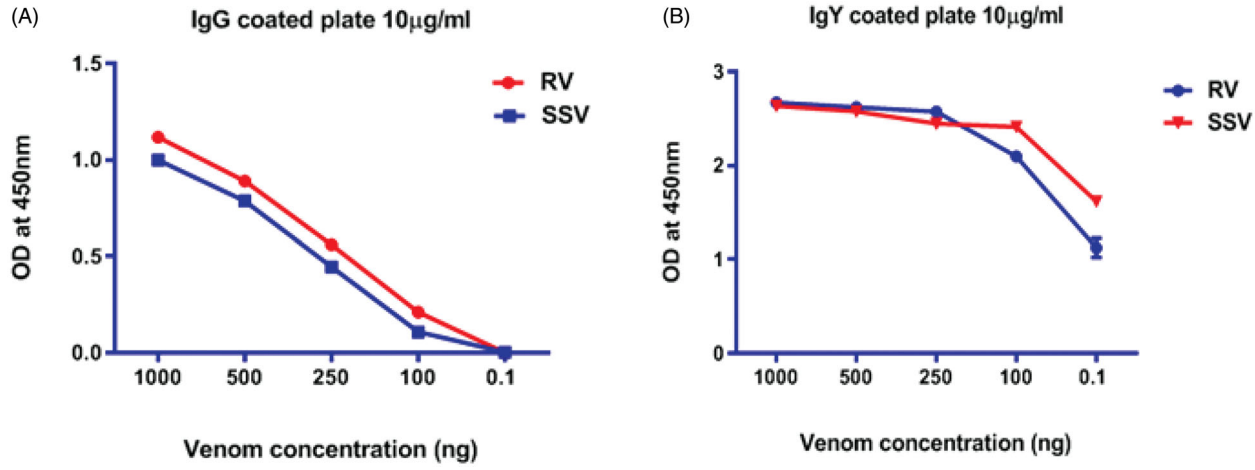

Figure 5. (A, B) Sandwich ELISA.

IgY antivenom, when serially diluted and added to the plates coated with the venoms of (RV, Cobra and Krait) showed cross reactions with the three medically important snake species as mentioned. The anti SSV IgY antivenom showed reactions of up to 1:1000 against the RV venoms whereas for the venom of cobra and krait the cross reactions occurred up to dilutions 1:500, whereas the anti RV IgY antivenom showed cross reactions with SSV venom up to dilutions of 1:500 and against the venom of cobra and krait the dilutions ranged up to 1:100 (Figure 6(i)). The IgG antivenom against both the venoms showed higher cross reactions when compared to that of the IgY antivenom. The anti RV and anti SSV IgG antivenom reacted up to dilutions of 1:1000 against the venom of SSV and RV respectively, whereas for cobra and krait venom the cross reactions were observed up to dilution 1:500 (Figure 6(ii)).

3.2. Neutralization potential of IgY, IgG, and commercial antivenom

The ability of the monovalent IgY and IgG antivenom to neutralize the lethality caused by the respective snake venoms was evaluated by the *in vivo* assay. For this purpose, groups of mice ($n = 4$) were divided into different groups and a challenge dose of venom ($3LD_{50}$) was incubated for 30 min at 37 °C with different concentrations of the IgY, IgG and commercial antivenoms respectively. The ED_{50} values can be expressed in terms of μl of antivenom neutralizing $3LD_{50}$ of snake venoms and are as follows: 96.66 μl of chicken IgY, 89.33 μl of rabbit IgG and 35 μl of commercial antivenom neutralized $3LD_{50}$ of *D. russelii* snake venom and that for *E. carinatus* venoms were 80.4 μl (IgY), 69.2 μl (IgG), and 40.8 μl (commercial antivenom). ED_{50} is also expressed as mg of venom neutralized/g of antivenom, and it was found that 1 g of

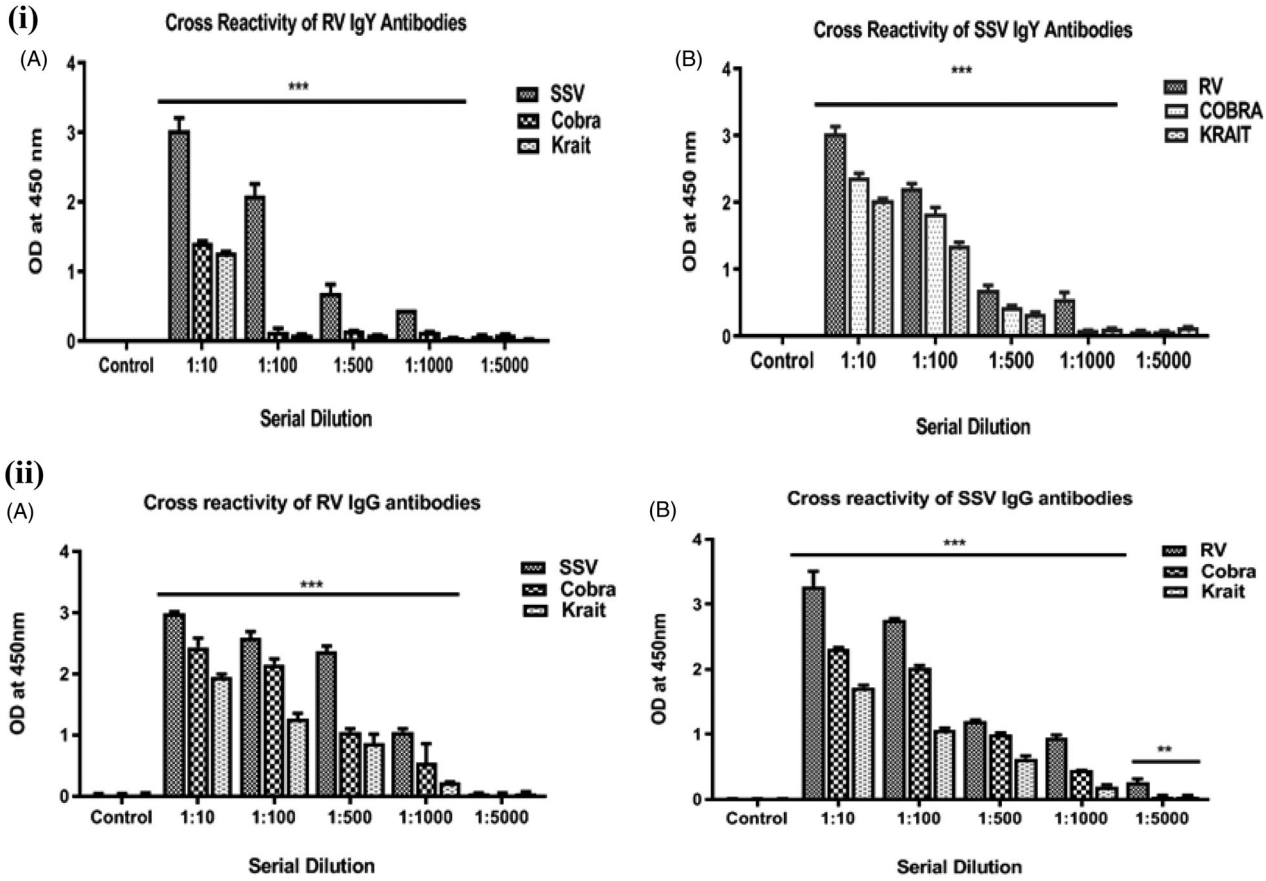

Figure 6. Cross reactivity analysis of raised anti SSV and anti RV IgY antibodies (i A, i B) and IgG antibodies (ii A, ii B). All samples were added in duplicates and the values are expressed in mean ± SD; significance with ANOVA Bonferroni's test. $^{***}p < 0.001$, $^{**}p < 0.01$.

chicken IgY, rabbit IgG and commercial antivenom neutralized 0.0104, 0.011, and 0.028 mg of *D. russelii* snake venom and 1 g of anti *E. carinatus* IgY, IgG and commercial antivenom neutralized 0.015, 0.017, and 0.030 mg of *E. carinatus* snake venom (Tables 1 and 2). The potency of antivenoms neutralizing mg of venoms were calculated as per the formula described in methods (sec 2.13). Each ml of IgY, IgG and commercial antivenom neutralized 0.3, 0.34, and 0.6 mg of *E. carinatus* snake venom and 0.2, 0.22, and 0.57 mg of *D. russelii* snake venom (Figure 7).

4. Discussion

Antivenoms of equine origin is the currently available treatment administered to patients suffering from venomous snake bite in India. These equine derived antivenoms are polyvalent antibodies prepared against the venom of Big Four snakes of India viz., Russell's viper, Saw Scaled viper, Cobra and Krait. In Asia the bites occurring from Russell's viper snake results in highest mortality, of which countries such as Myanmar and Sri Lanka suffer from 40% and 30% of RV snakebite incidences, respectively, whereas in western India 20.8% of cases have been reported

(Kalita *et al*. 2018). Envenomation caused by the Indian viper (RV and SSV) venoms requires immediate medical care and are considered as the category I medically important snakes (Patra *et al*. 2017, Kalita *et al*. 2018). The geographical difference between the snake venoms of Indian origin (i.e. North, South, East and West) has laid a serious concern for effective designing and manufacturing of snake antivenoms in India (Kalita *et al*. 2018). The adverse reactions caused by the present antivenom has led researchers look for an alternative method for producing antivenoms (Tanwar *et al*. 2017). Recently Tanwar *et al*. 2017 generated antivenom in camels against the venom of *Echis carinatus sochureki*, which is considered as a medically important snake in north western India (Rajasthan) and currently there is no commercial antivenom available for the particular snake venom. Similarly, Joseph *et al*. 2007 reported envenoming incidences by *Hypnale hypnale* (Hump nosed pit viper) in southern India (Kerala), for which specific antivenom is currently unavailable. In the present study, we

Table 1. Concentration of antivenom antibodies to neutralize 3 LD_{50} of *D. russelii* venom.

Snake species	Venom LD_{50}	Challenge dose	Antivenom species	Volumes of antivenom protecting 50% of mice from lethality of venom ED_{50}(µl/mice) (95% CI)	Concentration of antivenom antibodies (mg) neutralizing 3 LD_{50}	mg of venom neutralized by g of antivenom (mg/g)	Protein concentration (mg/ml)
Daboia russelii	10µg	3 LD_{50}	Chicken	96.66 (88.02–105.30)	2.88	0.0104	30
			Rabbit	89.33 (82.51–96.15)	2.67	0.011	
			Equine	35.00 (29.34–40.65)	1.05	0.028	

Table 2. Concentration of antivenom antibodies to neutralize 3 LD_{50} of *E. carinatus* venom.

Snake species	Venom LD_{50}	Challenge dose	Antivenom species	Volumes of antivenom protecting 50% of mice from lethality of venom ED_{50}(µl/mice) (95% CI)	Concentration of antivenom antibodies (mg) neutralizing 3 LD_{50}	mg of venom neutralized by g of antivenom (mg/g)	Protein concentration (mg/ml)
Echis carinatus	12 µg	3 LD_{50}	Chicken	80.4 (73.42–83.37)	2.4	0.015	30
			Rabbit	69.2 (62.77–75.62)	2.07	0.017	
			Equine	40.8 (35.34–46.25)	1.2	0.030	

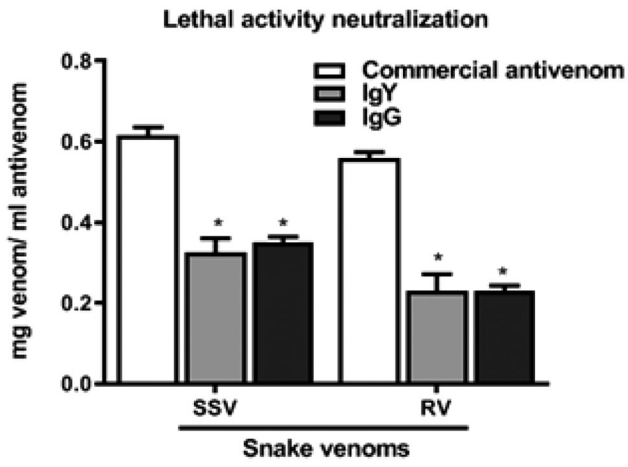

Figure 7. Lethal activity neutralization: The capacity of the commercial antivenom, IgY and IgG antivenoms to neutralize the lethal activity of SSV and RV venoms, corresponding to 3 LD_{50}. The antivenoms were administered intravenously in Swiss albino mice and the lethality was calculated from the number of deaths within 48 h. The ED_{50} was calculated as mg of venom/ml of antivenom. There was a significant difference between the commercial antivenom and IgY antivenom and commercial antivenom and IgG antivenom ($p < 0.05$). Statistical analyses were performed with one-way ANOVA (Tukey's multiple comparison tests). $^*p < 0.05$.

attempted to look for alternative animal or bird model for the production of snake antivenom against the venoms of *D. russelii* and *E. carinatus* and compared with the commercially available equine antivenom.

Prior to immunization, the characterization of the snake venom was done by SDS-PAGE analysis (Figure 3(ia,iia)). The lethality of the snake venom was checked in swiss albino mice as per the protocol of Camey *et al*. 2002. The LD_{50} for *E. carinatus* venom and *D. russelii* was found to be 12 µg and 10 µg respectively. In order to produce monovalent antivenom against the venom of *E. carinatus* and *D. russelii*, we immunized chickens and New Zealand white rabbits subcutaneously at multiple sites and booster doses were given in a similar manner at an interval of 15 days (Tables 1 and 2). The production of IgY antibodies represents several advantages over the mammalian IgG which has been proved widely (Almeida *et al*. 2008, Meenatchisundaram *et al*. 2008, Araujo *et al*. 2010, Liu *et al*. 2017, Lee *et al*. 2017). Followed by immunization of snake venom in chickens, the eggs were collected and were purified by the method of Polson *et al*. 1980. The purified IgY antivenom antibodies showed a high titer of 1:32,000 and 1:64,000 for RV and SSV venom respectively after 90 days post immunization which was achieved by administration of booster doses (Figure 1(A,B)). A similar result was observed by Almeida *et al*. 2008 against the venoms of *Bitis* and *Naja* sps. The molecular weight of IgY in non-reducing conditions shows a single band at 180 kDa, whereas under non- reducing conditions, two bands are observed at 65 kDa and 25 kDa respectively (Liu *et al*. 2017). The purity of the IgY antivenom was analyzed by SDS-PAGE (10% gel) under reducing conditions showing bands at 65 kDa and 25 kDa respectively (Figure 2(A)). The specificity of the IgY antivenom against the particular venom was evaluated by Western blot analysis. The IgY antivenoms (anti SSV IgY and anti RV IgY) were found to recognize the proteins of the specific snake venom (Figure 3). Irrespective of the advantages IgY antibodies has shown in the past, it is scarcely used (Lee *et al*. 2017). The quantity of antibodies produced by 63 chickens is equivalent to the same amounts of antibodies which a

horse produces each year (Navarro *et al*. 2016). An important aspect related to snake antivenoms is its affordability in the low-income countries (countries of Sub-Saharan Africa and Asia), which shows a big difference in the treatment of snakebites. Chicken IgY antivenoms can be produced at rates much cheaper to equine antivenoms and additionally it does not activate the mammalian complement system (Schade *et al*. 2005, da Rocha *et al*. 2017).

To further compare the generated monovalent IgY, monovalent IgG against the venom of RV and SSV were generated by immunizing rabbits (Table 2). The purity of the IgG antivenom antibodies was checked by SDS PAGE analysis under reducing conditions which showed bands at molecular weights (75 kDa and 25 kDa, respectively) (Figure 2(B)). The specificity of the antivenom antibodies were evaluated by western blot analysis, which detected the snake venom proteins effectively (Figure 3(iib)). Venkatesan *et al*. (2014) produced IgG (from rabbits) type of antisera against the venom of *Naja naja naja* and tested the ability of its neutralization by administering cocktail of antisera raised against different proteins of snake venom and compared with that of the IgG whole antivenom and commercial antivenom. Beghini *et al*. (2008) showed the ability of rabbit antiserum to neutralize the neurotoxic, myotoxic and phospholipase activities of crotoxin purified from *Crotalus durissus cascavella* snake venom.

Detection of snake venoms remain a mainstay for effective therapy of snakebite. At present there are no snakebite detection kits available in India (Bawaskar and Bawaskar 2019). Commonwealth Serum Laboratories (Bio CSL, Australia), only has the ELISA based detection kit, which effectively detects the five snake venoms of Australia within 30 min (Shaikh *et al*. 2017). In the same context, for the Indian subcontinent (Pawade *et al*. 2016, Shaikh *et al*. 2017) in their published research work have demonstrated the use of dot ELISA for detection of venom of Big four snakes of India. In the current research work, we found that IgY antivenom antibodies could detect the snake venoms up to range of 0.1 ng (Figure 5(B)), whereas the IgG antivenom was able to detect the venom at concentration of up to 100 ng effectively (Figure 5(A)). The drawback of the current sandwich ELISA is the time taken for detection (3 h). Time for detection could be reduced by conjugating the monovalent antivenom with HRP enzyme. Cross reactivity of the antivenoms also plays an important role for detection of snake venoms. In order to find out, whether the monovalent antivenoms from chicken and rabbits do react with the other venoms, we performed ELISA for the antivenoms against the Cobra and Krait venoms (Figure (6i,ii)). It was obvious that the antivenom (anti SSV IgY and IgG) would cross react with that of the *D. russelii* venom, as these venoms share some common proteins and enzymes. It was found that the anti SSV IgY and IgG antivenom had cross reactions with that of the *D. russelii*, *Naja naja* and *B. caerulus* venoms at dilutions up to 1:1000, 1:500, and 1:500 respectively (Figure 6(ib,iib)). The anti RV IgY antivenom antibodies showed cross reactions against the venom of *E. carinatus*, *Naja naja*, *B. caerulus* at concentrations up to 1:500, 1:100, and 1:100 respectively, whereas the anti RV IgG antivenom showed reactions at dilutions 1:1000, 1:1000, and 1:500 respectively (Figure 6(ia,iia)).

Relative avidity ELISA was performed to evaluate the binding ability of the venom and antivenom. Ammonium thiocyanate in different molar concentrations (0, 2, 4, 6, 8 M) was used for this assay which is a potent protein disruptor. When compared to the IgG and the Commercial antivenom, IgY antivenom was less stable and a reduction in the OD value was observed at 4 M concentration, whereas the IgG and commercial antivenom were stable up to 6 M concentration (Figure 4(A,B)). The venom antivenom stability was directly proportional which was observed in the *in vivo* neutralization assays. For neutralization assays, we followed the method of pre incubation assays whereby $3LD_{50}$ challenge dose of the venom was incubated at 37 °C for 30 min with different concentrations of the antivenoms (IgY, IgG and commercial antivenom). The ED_{50} was calculated as mg of venom neutralized/ml of antivenom. Each ml of IgY anti SSV antivenom neutralized 0.3 mg of venom, whereas each ml of IgG and commercial antivenom neutralized 0.34 mg and 0.6 mg respectively of SSV venom. Each ml of anti RV IgY, IgG and commercial antivenom neutralized venom at concentrations 0.2, 0.22, and 0.57 mg of venom, respectively (Figure 7). In this study, it was clear that the commercial antivenom neutralized the lethality of respective snake venom at concentrations lower than that of the IgY and IgG antivenoms. Recently, Senji Laxme *et al*. (2019) stated that the currently available Indian antivenom which is said to be specific against the Big Four snake venoms, was not effective in neutralizing *B. caeruleus* venom from the northern regions. There are few drawbacks from the antivenom produced from equines such as the production cost, purity of pure and specific IgG. Chicken IgY could become an alternative source for equine antivenom by improving the immunization process, which is directly proportional to production

of high concentration of antivenom antibodies. These IgY antivenom antibodies could replace equine antivenoms as these antivenoms are most likely not to cause any adverse reactions upon administration (Sifi *et al*. 2018).

5. Conclusion

In conclusion, for the first time a comparative study was performed with that of the chicken IgY, rabbit IgG and commercial antivenom. This research work confirms the possibility of the use of chicken egg yolk antibodies as a possible alternative to equine antivenom. Chicken egg yolk antibodies are an easy to obtain source for the production of polyclonal antibodies and would be an excellent substitute for mammalian IgG antibodies. However, further studies and pre-clinical trials must be conducted at an international level, prior to bringing chicken IgY antivenom antibodies as an alternative to the equine antivenom.

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Ethical approval

All experiments involving the use of animals were ethically approved by the Institutional Animal Ethical Committee (IAEC) at PSG Institution of Medical Science and Research, Coimbatore with proposal approval No. 385/2018/IAEC and 402/2018/IAEC.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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