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DEVELOPMENT OF A SANDWICH ELISA FOR THE DETECTION OF RUSSELL'S VIPER VENOM USING CHICKEN EGG YOLK ANTIBODIES (IGY)

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ABSTRACT

Envenomation from snakebite is considered as a serious health hazard in tropical and sub tropical countries. Detection of venom / venomous bite is still a major concern for the treatment of the patients affected by snake bite. Hence in this study we produced antivenoms from chicken and rabbits and used for the development of sandwich ELISA for the detection of russell's viper venom. Hyper immune eggs were purified by the method Polson et al., 1980. Titration of the egg yolk sourced antibodies was found to be 1:10000 whereas antisera from rabbits had high titres (1:14000) after the 56th day of immunization. These antibodies were further used for the detection of venom of russell's viper and plate coated with IgY antibodies were able to detect the snake venom at a range of 10-300ng of samples. IgY antibodies can be used as an alternative source for equine derived antibodies as chicken derived antivenom antibodies can be produced easily in large amounts and are reported widely for its use as antivenom.

KEYWORDS: Envenomation from snakebite is considered as antivenom.

1. INTRODUCTION

Venom from snakes is a highly modified saliva containing zootoxins used by snakes to immobilize and digest the prey. Envenomation from snakebite is considered as a serious health hazard in tropical and sub tropical countries. There are around 1.8 – 2.7 million people being affected from envenomation globally every year (Gutierrez, 2018). Financial and commercial factors play an important role in saving the lives of the patients. High treatment costs, reduced productivity of the antivenoms and low cost income impacted families suffer

from this crisis throughout their lifetime. There are four common venomous snakes in India, which are responsible for causing major damage to the humans and are named as the Big Four snakes of India which comprises of Indian Cobra (*Naja naja*), Krait (*Bungarus Caerulus*), Russell's viper (*Daboia russellii*) and Saw Scaled viper (*Echis carimatus*). The only treatment available for patients envenomated by snakebite is the polyvalent antivenom produced from horse plasma against the Big Four snakes of the country (Kumar et al., 2005). According to Bawaskar (2004) 5 lakh vials of antivenoms are being supplied throughout the country to overcome from the deadly envenomation. Many patients suffer due the adverse reactions caused by the antivenoms and also because of the huge amounts of the antivenoms being injected into the patients. At present there are no detection kits available in India to detect the presence of venom in the patients and also of identifying the species of snake. This work focuses on the production of the antivenom antibodies in chickens and rabbits and their efficacy evaluation by means of a sandwich ELISA.

1. MATERIALS AND METHODS

1.1. Venom

The venom of Russell's viper was obtained from Irula Snake catcher's society, Chennai in lyophilized form and was stored at -20ºC until use. The stock solution of the venom was prepared using 0.9% saline (1mg/ml) and was used for performing the immunoassays.

1.2. Chicken Derived Antivenom Antibodies

White leghorn chickens were purchased from a local farm and were kept in individual cages with proper food and water *ad libitum*. The chickens were then immunized intramuscularly with sub lethal doses of venom and the sensitizing dose was administered using Freunds Complete adjuvant whereas the booster doses were administered using Freunds Incomplete adjuvant. Followed by immunization, the eggs were properly labelled and collected and stored at -20ºC until use.

1.3. Purification of the IgY antivenom antibodies

Egg yolk derived antivenom antibodies were purified using the method of Polson et al., 1980. Briefly the eggs were broken and the yolk was transferred into the centrifuge tubes, Phosphate Buffered Saline (pH- 7.2) was added to the tubes. Followed by mixing, PEG 6000 was added and centrifuged at 10g for 20 mins at 4ºC. On centrifugation, the supernatant was collected and filtered and added with PEG (8.5%) and again centrifuged as mentioned above. The final centrifugation was performed with 12% PEG and the final pellet was dissolved in

the required volume of PBS (~ 1ml) and was further purified by dialysis and DEAE Ion Exchange column chromatography.

1.4. Titration of the antibodies (IgY)

Indirect ELISA was performed to check the titre values of the antibodies. Polystryene microtitre plates were coated with venom (1µg/100µl/ml), added with the carbonate buffer and incubated overnight at 4ºC. Followed by incubation, the plates were washed thrice with PBS containing 0.05% Tween 20. The raised antivenom antibodies were serially diluted and added to the plates followed by incubation at 37ºC for 1 hour. Secondary antibodies (Rabbit anti chicken IgY HRP) were added to the plates after the plate was washed with PBST thrice. Secondary antibodies were diluted at a concentration of (1:1000) and the plate was incubated as mentioned above. Plates were again washed and incubated with substrate (TMB) for 20 mins in dark at room temperature. The reactions were stopped by the addition of 4N sulphuric acid and the readings were observed at 450nm.

1.5. Rabbit antisera: Immunization and Purification

New Zealand white rabbits were immunized subcutaneously at multiple sites with sub lethal doses of venom with Freunds complete and Incomplete Adjuvants as mentioned above. The titration of the antivenom was checked through Indirect ELISA after booster injections were administered. Rabbit sera was collected and stored at -20ºC until use.

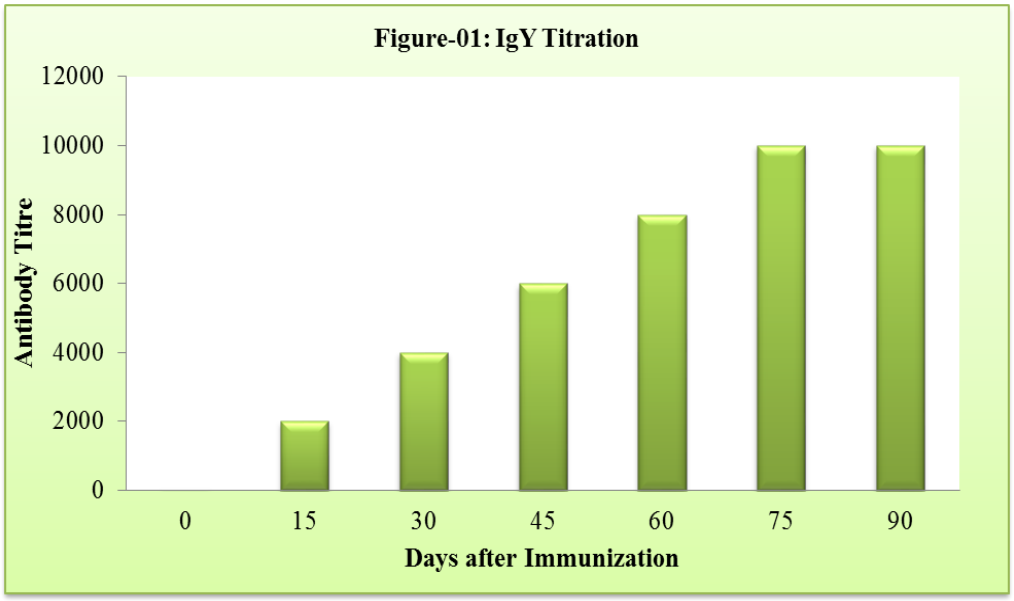
1.6. Titration of serum antibodies: Collected and stored antisera were subjected to Indirect ELISA as mentioned above for the titration of chicken antibodies. Secondary antibodies used were Goat Anti Rabbit IgG HRP at a concentration of 1:1000.

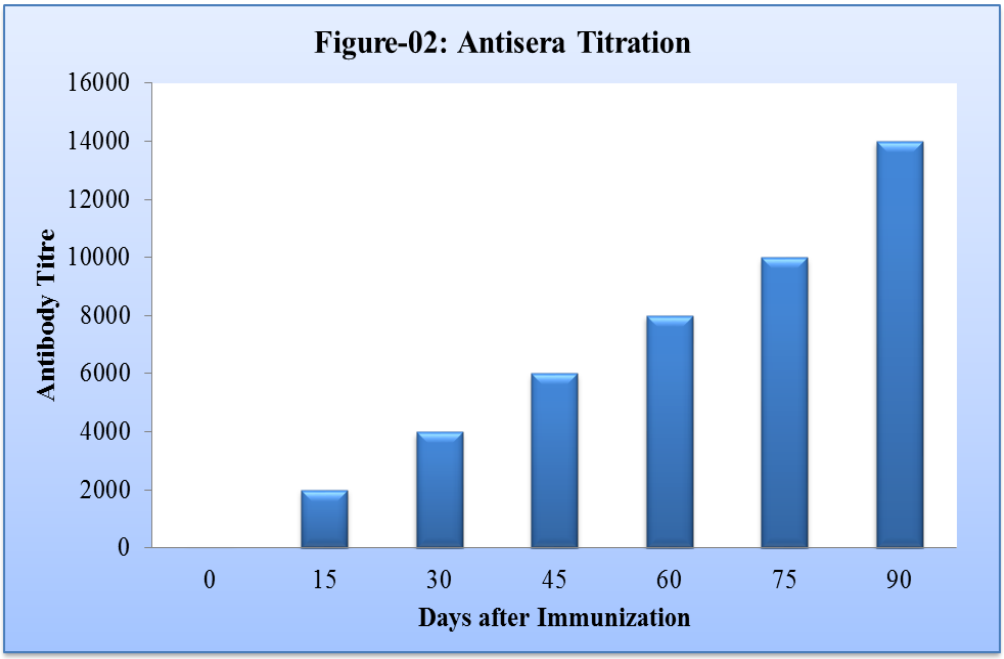
1.7. Sandwich ELISA for the detection of snake venom: For the development of ELISA for the detection of snake venom, we performed Double Antibody Sandwich (DAS) assay for the detection of Russell's viper venom. Chicken derived antivenom (IgY) antibodies (100ng) were coated on the plates based on their optimal concentrations with coating buffer (100µl) and incubated overnight at 4ºC. The plate was washed thrice with washing buffer (PBS containing 0.05% T-20). Blocking of the unbound antibodies was done using BSA (1%) (200µl/well) and incubated at 37ºC for an hour. The plates were again washed with wash buffer and venom (R.V) was added in different concentrations (10ng-300ng) and incubated at 37ºC for an hour. Plates were washed as mentioned above and followed by that rabbit antisera for Russell's viper venom was (100ng) was added to each well (100µl/well) and was

incubated at 37ºC for an hour. The plate was washed thrice and followed by washing the secondary antibody (Goat Anti Rabbit IgG HRP) was added at a concentration of 1:1000 diluted in PBST buffer and added to wells (100µl/well) and incubated at 37ºC for an hour. The plate was washed thrice with the wash buffer and substrate (TMB) was added to individual wells (100µl) and kept for incubation in dark for 15 mins. The reaction was stopped with stop solution (4N H_2SO_4) (100µl/well). The absorbance was read at 450nm.

2. RESULT

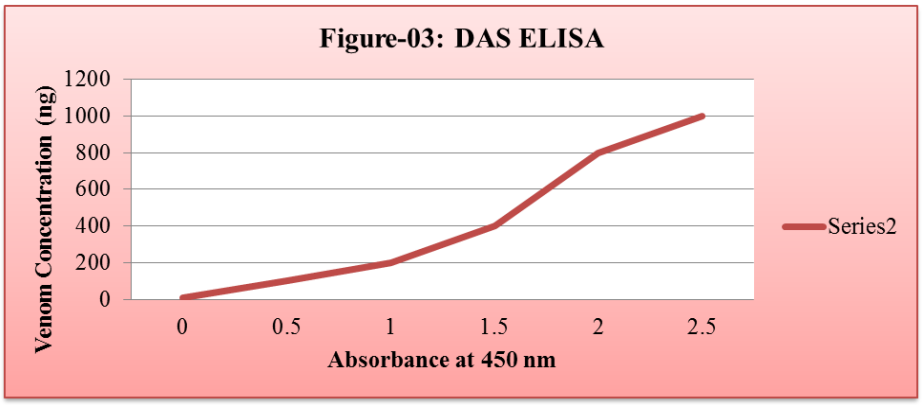
2.1. Purification and characterization of antivenom IgY antibodies: The production of the antibodies starts with a host (mostly animals and birds) and in this case we used chickens and rabbits for the production of antivenoms against the venom of Russell's viper. White leghorn chickens immunized with sub lethal doses of venom with Freunds Complete and Incomplete adjuvants at multiple sites following the method of (Chotwiwatthanakun et al., 2001) and resulted in high titration of antivenom antibodies reaching a peak of 1:10000 after 8 weeks of immunization. These antivenom antibodies have the potential to recognize the venoms even at much lower concentration. The high titre eggs were purified using the method of Polson et al., 1980 with some modifications. The purified IgY antibody was subjected to dialysis and followed by that, the antibodies were column purified to remove the impurities. The concentration of the antibodies was checked using spectrophotometer at 280 nm. Indirect ELISA was performed in order to check the titration of antibodies and it was found that after the 63rd day of immunization, IgY antibodies reached a peak titre of 1:10000 and remained constant for upto 120 days post immunization. Immunization was carried out and whenever the titre levels reduced, the chickens were immunized with lower concentration of venom with Incomplete adjuvants, thereby ensuring the continuous production of the specific antibodies.





2.2. Purification and characterization of antisera: New Zealand white rabbits were used for the production of antisera for Russell's viper venom. Rabbits weighing around (2-2.5kgs) were immunized subcutaneously. Bleeding from marginal ear veins was performed as and when the rabbits were immunized with booster doses of venom. Indirect ELISA was performed to check the titration of the raised antivenom antibodies and it was found that rabbit antisera was effective and reached a high titration of 1:14000 post immunization after 60 days post immunization. These antivenom antibodies were then stored at -20ºC for further use.

2.3. Double Antibody Sandwich (DAS) ELISA: DAS ELISA was performed as mentioned briefly in materials and methods. Chicken derived antivenom antibodies were effective in capturing the antigen, in this case (Russell's viper venom) at a concentration of 100ng which is quite promising when used as a diagnostic tool for detection of snake venom.



3. DISCUSSION

The most conventional method for the production of antibodies is the immunization of a suitable host (Lee et al., 2017). Early stages of immunization marks the production of onset of IgM antibodies which in later stages and due to class switching which occurs in later stages of B cell development leads to the production of IgG type of antibodies. Chickens provide a better alternative when compared to the equine immunoglobulins, as IgY antibodies can be produced from the yolks of the eggs immunized with the particular antigen. Recently it has been shown that transfer of the IgY from serum to egg yolk is a receptor mediated process which allows a selective transfer of antibodies from the maternal serum (Muller et al., 2015). Chicken derived antivenom has been widely published (Almeida et al., 2008; Meenatchisundaram et al., 2008; Araujo et al., 2010) has proven the use of IgY antibodies in neutralizing the venom. Detection of the venom remains a mainstay and also the need of the hour as based on the identification of the snake, antivenoms are administered. Brunda et al., 2006 successfully reported the use of chicken as well as rabbit antibodies for the detection of venom in samples from patients. Chicken IgY also presents several advantages over mammalian antibodies as IgY presents low costs, high yield and preventing animal bleeding. Molecular weight of IgY is approximately 167 kDa and isoelectric point varies between 5.7 and 7.6 (Almeida et al., 2008).

ELISA is a sensitive technique to find out the antibody titers during immunization period and also to determine antibody activity during different purification processes. More generally,

this *in vitro* test can be satisfactorily used for monitoring activities and to carry out determinations at intermediate steps of manufacturing processes. In ELISA a highest titre of 1:10000 was observed during 56th day of observation. These results were comparable to the work done by Almeida *et al.* (1998) who showed that antibodies against venom components started to appear in serum two weeks after immunization began and reached high titre at 45th day and remained stable till 168th day observation. The chicken egg yolk antibodies are present in the egg for upto 100 days after the immunization (Mayadevi, 2002). Rungsiwongse *et al.* (1991) reported a significant correlation between their ELISA and the antivenom potency tested *in vivo* against *Naja naja* venom. Heneine *et al.* (1998) have shown a good correlation between ELISA and the *in vivo* potency of Bothropic antivenoms when crude Bothrops venom was fractionated and the purified fractions were used as the antigen for ELISA.

Our study proves that chicken derived antivenom antibodies and rabbit antisera are potential diagnostic antibodies which could be taken further for the development of a rapid detection kit for the detection of snake venom.

4. CONCLUSION

Envenomings from snake bite is a serious health hazard and a neglected tropical disease, affecting millions of people worldwide every year. A major concern is the regional variation of the snake venom from species to species, which is the major drawback in the production of specific antivenom. Detection of snake bite is considered to play a most important role, which can save the lives of envenomated patients. Developing effective antivenom is a global challenge and a proper method for the production of antivenom should be encouraged. Novel Preclinical tests are required at *invitro* level so that the use of animals can be made to a minimum percent.

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