CHAPTER 6

Critical Discussion and Future Prospects

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6.1 Critical Discussion

Antivenom administration still remains the pillar of modern therapy for snakebite envenomation, however, the management of snakebite is a multidimensional process which involves not only medical support but also other aspects such as training, diagnosis, logistics and financial support [273,274]. Various limitations are associated with the currently available antivenom and there is a need to develop more effective antivenom which are safe to use, stable, with cross-species neutralization capacity and also financially feasible for the patients [133,266,275]. In a country like India, it is difficult to procure venom in quantities required for antivenom manufacturing, since captive breeding of snakes is not allowed as they are protected under Schedule 11 of the Wild Life (Protection) Act 1972 [276]. Advances in technological innovation and our understanding of these issues provide an opportunity for reconsidering the steps associated with development and manufacturing of antivenom. The emergence of new molecular formats and innovative approaches may be engaged and utilized for improving the therapeutic efficacy of antivenom for snakebite management [277]. In this chapter, some of the approaches to overcome the limitations of currently available polyvalent antivenom as well as the future prospects of the findings from this study have been discussed.

6.1.1 Steps to consider before antivenom manufacturing

Some important considerations before beginning the complex steps associated with polyvalent antivenom manufacturing have been recently highlighted by Ratanabanangkoon [80]. Firstly, the number of snake species to be included should not exceed 5-6 species, as including venom from more species may result in a low yield of antibodies which would be ineffective against some of the venom proteins available in the venom pool due to an effect called ‘antigenic competition’ [278,279]. Secondly, if the lethal toxin fractions of various snake species are known, then the

hyperimmunogenic non-lethal fractions can be separated out from the venom pool used for the immunizing the animals. This may lead to use of venom pool from up to 12 snake species, and thus antivenom with improved efficacy and paraspecificity can be produced [156,280,281]. Thirdly, the synergistic and antagonistic interactions between the venoms used for antivenom manufacturing should be considered. For instance, the efficacy of antivenom raised against *Bothrops asper* venom is lowered by the presence of *Crotalus durissus ruruima* and *C. simus* venom in the venom pool. Similarly, the presence of some venoms act as stimulants during the production of polyvalent antivenom against *Bothrops* and *Crotalus* [282]. Fourthly, mixed polyvalent antivenom with a broad paraspecificity can be produced by mixing the whole immunoglobulin or their fragments from two separate polyvalent antivenom raised against the venoms of different snake species. For instance, a mixed polyvalent antivenom was successfully tested in Sub-Saharan Africa against venoms from eleven snake species, which was a mixture of one antivenom raised against five *Echis* and *Bitis* species, and another raised against six elapid species [279]. Further, Chakma et al. have highlighted that health and hygiene of animals, like horses, mules and donkeys, used for antivenom production play a major role in effectiveness of antivenom. Inoculation methods of venom and purification process of snake venom specific antibodies are also important factors for producing high quality antivenom [276].

6.1.2 Region-specific antivenom

Production of region-specific polyvalent antivenom have been advocated by various authors [74,283,284] to address the issue of venom variation in different snake species due to which currently available antivenom are rendered ineffective or partially ineffective. In this approach, the polyvalent antivenom is developed targeting the venom pool of all the medically important representative snakes of a particular region. However, implementation of such approach also has various challenges, such as the selecting representative venom samples and their reproducibility. Although in the short term such antivenom may be effective, but in the long term the availability of representatives and reproducibility of the venom samples remain a challenge. In order to overcome this issue, long-term monitoring and standardized manufacturing system is required so that the major components of the venom pool is maintained [283].

6.1.3 Alternate approaches currently explored

6.1.3.1 Single-chain variable fragments (scFvs)

Among the most widely explored recombinant antibodies is the Single-chain variable fragment (scFv), which is expressed as a single polypeptide chain consisting V_L and V_H fragments of IgG linked by a 15 amino acid peptide. It has been reported in numerous studies to exhibit inhibition of various snake venom toxins both in vitro and in vivo. For instance, Castro et al. have reported recombinant scFv obtained from monoclonal antibodies to completely neutralize a snake venom metalloprotease (BaP1) induced toxicity from *Bothrops asper*, such as muscle necrosis, hemorrhage and inflammation in vivo [285]. Another study by Roncolato et al. using human monoclonal antibody derived scFv fragment capable of inhibiting PLA_2 activity of *Bothrops jararacussu* to exhibit partial inhibition of in vitro PLA_2 activity from three conspecific venoms of *B. jararaca*, *B. moojeni* as well as *B. neuwiedi*. Assays using the scFv fragments lowered myotoxicity and enhanced vitality of experimental models injected with *Bothrops* venom.

6.1.3.2 Single-domain antibodies (sdAbs)

The currently available polyvalent antivenom are based on polyclonal IgG antibodies or $F(ab')_2$. Newer single domain antibody fragments (sdAbs) which consists of only one domain is being investigated to neutralized snake venom proteins. Among the sdAbs particular focus is on the heavy-chain variable domain (V_HHs) also known as nanobodies [286]. These heavy chain domains occur naturally in camelids and have a similar neutralization capacity compared to existing antivenom [287]. However, they provide two major advantages over currently available antivenom, firstly, these sdAbs are 10 times smaller than IgG molecules and have a long flexible loop which may enable them to penetrate deeper tissues at a lesser time and access buried epitopes as well [288], and secondly, they have a higher solubility and thermal stability, and also have a potential to fold back to their active form after thermal denaturation [289].

6.1.4 Next generation antivenom

The emergence of different antibody formats have provided an opportunity to develop robust mechanisms for better management of snakebite envenoming, however, some

important considerations should be accounted for before proceeding ahead. For instance, Thumtecho et al. suggested that the newer antivenom types should have a higher clinical efficacy than that provided by the current ones. Use of approaches such as toxicovenomics (which studies the toxicity of individual venom proteins) and antivenomics (which identifies the venom proteins which are non-immunodepleted or poorly-immunodepleted by the commercial antivenom) can be utilized in this regard to isolate the venom protein of interest or these can also be recombinantly expressed, and then these venom proteins can be utilized to search for neutralizing agents through targeted drug-discovery approaches [290–293]. Recombinant antivenom which is based on human monoclonal or oligoclonal mixture of IgGs have been explored which may have the benefit of being compatible with the immune system of humans and the cost of production is also estimated to be much lower compared to traditional antivenom [294]. Some of the formats of recombinant antivenom are briefly discussed below.

6.1.4.1 Monoclonal antibodies

Monoclonal antibodies are the primary recombinant antibody type explored for neutralization of snake venom proteins. Various studies have reported monoclonal IgGs to have neutralized snake venom proteins. For instance, Frauches et al. have reported monoclonal antibodies raised against PLA_2s, metalloproteases and thrombin-like enzymes to have neutralized the toxic effects of *Bothrops artox* venom injected in Swiss mice [295]. Subsequently, Schneider et al. also reported monoclonal antibodies against P-I metalloproteases (Atroxlysin-I) from *B. atrox* venom which had inhibitory activity against the in vivo hemorrhagic and proteolytic activity of the crude venom or Atroxlysin-I [296]. However, monoclonal antibodies target only specific venom toxins and since snake venom is a blend of various toxins, hence, a pool of monoclonal antibody targeting various toxins may be required for effective neutralization of snake venom toxins.

6.1.4.2 Human Monoclonal antibodies

Human monoclonal antibodies are specific antibodies developed by using hybridoma technique or phage-display technique [297]. These antibodies are designed to overcome two important limitations of monoclonal antibodies and sdAbs, the first limitation is the poor capability to neutralize different variants of the specific target they are designed

against, in other related species, and second is to avoid the immunogenic responses elicited by them [298]. Recently, Khalek et al. used a synthetic human monoclonal antibody (Fab) type called broadly neutralizing antibodies (bnAbs) which can neutralize a 3FTx type called α-neurotoxins, which is mostly abundant in elapids such as cobras, kraits and mambas. The bnAbs target the conserved regions present in the active site of these α-neurotoxins and disrupt their functioning [298]. These types of antibodies have been extensively studied in case of viral diseases, such as HIV [299–301]. Through a two-step selection strategy, a high-throughput subset of antibodies were selected which could bind to the epitopes conserved in α-neurotoxins from different snake species. The antibodies were synthesized and the best candidate with highest affinity was selected as the lead antibody (95Mat5). Structural characterization was performed to understand its binding mechanism. Pre-incubation of the antivenom provided in vivo protection in mice against lethal challenge of α-neurotoxin rich crude venoms (*Naja kaouthia*, *Dendroaspis polylepis, Ophiophagus hannah*) and α-Bungarotoxin (*Bungarus multicinctus*) of Asia and Africa. Using this strategy, broadly neutralizing antibodies can be developed targeting the conserved regions of different snake venom families, which can be combined to develop a universal antivenom for effective snakebite management. These antibodies, since derived for human cells, provide the advantage of not eliciting any side-effects as observed in traditional animal derived antivenom [298].

6.1.4.3 Protein based ADDomer nanoparticles (The ADDovenom project)

The European Union funded ADDovenom project seeks to produce next generation antivenom against two groups of African snakes *i.e.*, Saw-scaled vipers (*Echis* sp.) and Mambas (*Dendroaspis* sp.) using a protein-based scaffold which acts as an effective snakebite therapy at a low cost [302]. The ADDomer would be a megadalton-size nanoparticle whose structure is based on the protomer of the penton base protein of adenovirus. They can be recombinantly expressed where 60 protomers spontaneously assemble to 12 pentons called dodecahedron [303]. ADDomer-based nanoparticle has been previously utilized to produce effective vaccines against SARS-CoV-2, Chikungunya and foot-and-mouth disease [304–306]. The ADDomer based nanoparticles exhibit four major advantages over traditional antivenom, first, they are thermostable at temperatures ranging from -20 ^{o}C to 45 ^{o}C eliminating the need for cold storage, secondly, these can be stored in lyophilized form, which enhances the shelf life, thirdly,

these can be recombinantly produced in insect cell expression system with high yields, and finally, they have a low immunogenicity profile. This project aims to produce next generation antivenom which would be extremely safe, affordable and effective [302].

Exploring these newer antivenom types may help in addressing the current challenges associated with snakebite envenomation using antivenom.

6.2 Future prospects of this study

Identification and characterization of seven PLA_2 enzymes from *B. fasciatus* venom in this study may be utilized for future studies in this field. Some of these prospects are briefly outlined below:

- Improvement of the effectiveness of polyvalent antivenoms manufactured in India targeting the venom of *B. fasciatus* from Assam, India by inclusion of adequate amount of poorly-immunodepleted PLA_2 enzymes in the venom pool to raise antibodies against these proteins. A similar approach may be utilized to increase the effectiveness of these polyvalent antivenom against other medically relevant snakes of North-East India as well.

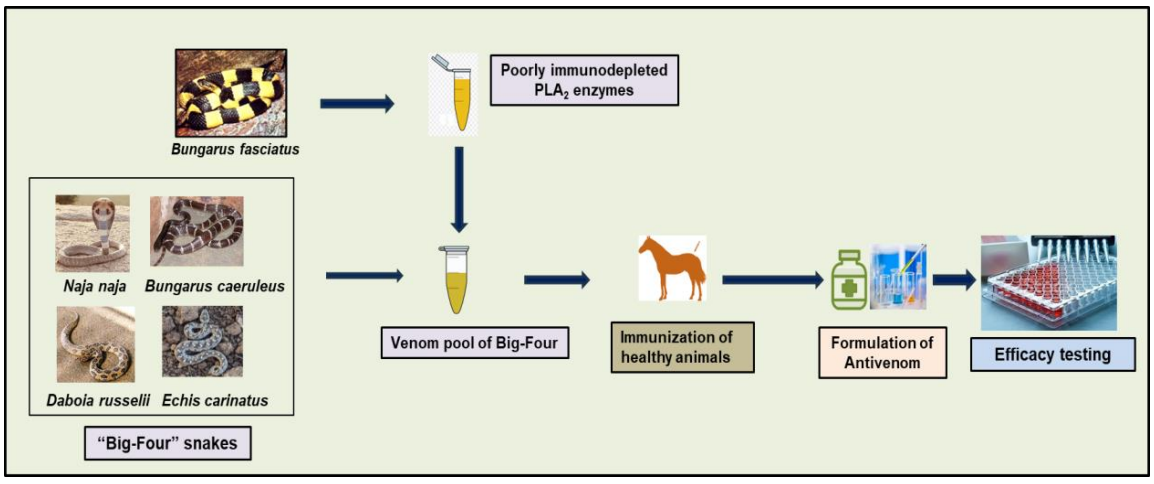

Figure 6.1: Flow-chart to improve efficacy of polyvalent antivenom manufactured in India against the venom of *B. fasciatus* from North-East India by inclusion of poorly-immunodepleted PLA_2 enzymes in the venom pool used for antivenom production.

- Determination of *in vivo* neutralization capacity of different polyvalent antivenom against crude *B. fasciatus* venom and poorly-immunodepleted proteins from various states of Eastern and North-East India in experimental animals.

- The B-cell epitopes (both conformational and linear) identified may be utilized for developing vaccines or toxin-specific antivenoms which specifically bind to these epitopes and neutralize their activity.

- Alternative to traditional antivenom: Design, synthesis and testing of inhibitors (eg. small molecular inhibitors, oligonucleotide aptamers, nanoparticles etc.) against the poorly-immunodepleted PLA_2 enzymes by utilizing the surface cavities identified in this study may prove to be effective alternatives to traditional antivenom therapy.