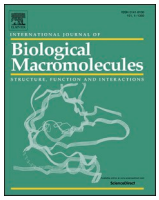
International Journal of Biological Macromolecules 240 (2023) 124478

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Review

Recent advancements in snake antivenom production

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ARTICLE INFO

Keywords:
Antivenom production
Neglected tropical diseases
Snakebite envenomation
BASE antivenom

ABSTRACT

Snakebite envenoming (SBE), a neglected tropical disease, claims lives of about 138,000 people globally, and antivenom is the only approved treatment worldwide. However, this century-old therapy has serious limitations, including limited efficacy and some side effects. Although alternative and adjunct therapies are being developed, their commercialization will take time. Hence, improving existing antivenom therapy is crucial for immediate reduction in the global SBE burden. The neutralization potential and immunogenicity of antivenoms depend primarily on the venom pool used for animal immunization and the production host, along with antivenom purification procedure and quality control. Enhancing antivenom quality and production capacity are also critical actions of the World Health Organization (WHO) roadmap 2021 against SBE. The present review details the latest developments in antivenom production, such as immunogen preparation, production host, antibody purification methods, antivenom testing-including alternative animal models, *in vitro* assays, and proteomics and *in silico* methodologies, and storage, reported from 2018 to 2022. Based on these reports, we propose that production of broadly specific, affordable, safe, and effective (BASE) antivenoms is fundamental to realizing the WHO roadmap and reducing the global SBE burden. This concept can also be applied during the designing of alternative antivenoms.

1. Introduction

Antivenom, also called antivenin and anti-snake venom (ASV), is the only approved treatment for snakebite envenoming (SBE) [1]. SBE is a neglected tropical disease (NTD) that occurs when a snake injects poisonous venom into a victim [2]. Annually, SBE is estimated to claim lives of 138,000 people worldwide [3]. Recently, the World Health Organization (WHO) devised a roadmap to reduce the SBE burden by half by 2030, and to achieve this goal, the critical actions include enhancements in antivenom quality and production capacity [4].

The conventional antivenom production system has stayed primarily unaltered for almost a century and suffers from a few major gaps [5]. For instance, the venom pool, which affects antivenom specificity, depends on animal age, habitat, diet, seasonal fluctuations, and venom composition of medically significant species [6,7]. This makes it challenging to manufacture since the venom pool is the vital raw material for production and impacts the specificity of the antivenom and batch variability. In addition, antivenoms usually cause adverse reactions in the recipient and are immunogenic. Also, they do not target all venom components, such as low molecular weight proteins, and antivenom raised against one species might not be effective against the other [8]. Further, they usually have low amounts of neutralizing antibodies and have an intricate manufacturing process. Thus, production protocols directly impact the specificity, safety, efficacy, and quality of the antivenoms. Hence improvements in production methods can be the stepping stones to partially or completely overcoming the abovementioned limitations and improving antivenom quality.

Over the years, the scientific community has consistently increased its interest in antivenom research, and antivenom production constitutes a significant part of recent antivenom research (Fig. 1). The present article aims to comprehensively review the latest advances in antivenom production processes published from 2018 to 2022. A search of the Scopus database till 01st January 2023, for 'articles' having the word 'antivenom' in the article title, abstract, or keywords fields, yielded 1010 articles published between 2018 and 2022, of which 4 were duplicate entries and removed. The abstracts of the remaining 1006 articles were manually screened, and articles pertaining to advancements in antivenom production were included in this study. One report was in Portuguese and was not included in the present review. Our analysis revealed that most antivenom production research is focused on generating alternative immunogens to either increase the species specificity or generate toxin-specific antivenoms. Development of antivenom

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https://doi.org/10.1016/j.ijbiomac.2023.124478
Received 10 February 2023; Received in revised form 11 April 2023; Accepted 12 April 2023
Available online 16 April 2023

testing assays is the second most researched topic. *In silico* tools for improving antivenom production are also emerging. The current developments in the choice of immunogens, production hosts, purification, and quality control are discussed and summarized in Fig. 2. We anticipate that this review article will benefit both inexperienced and seasoned researchers in this subject and anyone interested in learning about the current state of antivenom production and developments.

2. Immunization mixture

Conventional antivenoms are purified from the plasma of venom-immunized animals, and their efficacy is monitored for quality control [5]. Although it still forms the basis for the industrial manufacturing of antivenoms, several adjustments in this protocol have been suggested to improve the yield, potency, and stability of antivenoms. Major advancements in preparing the immunization mixture for antivenom production are discussed below.

2.1. Venom production

The first step in the traditional antivenom production process is venom collection *via* mechanical stimulation of the venom glands of wild or captive snakes [5]. However, repeated capturing of snakes from the wild can endanger their natural population. To overcome this, the use of captive snakes has been advocated [9]. The authors demonstrated that wild adults, long-term captive adults, and captive-born neonates of *Gloydius caucasicus* exhibited similar protein profiles. The wild adult and captive snakes had similar LD_{50}, hemorrhagic, and necrotic activities, except for edema activity, which might be dissimilar due to genetic or population differences. Further, the antivenom produced from captive adults venom effectively neutralized wild adult venom. Together these results reinforce the suitability of the captive adult snakes in generating venom pool for antivenom production. While captive snakes are an excellent source of venom, snake maintenance in captivity can be difficult. The nutritional adequacy and the cage substrate can significantly impact animal's survival probability and venom yield in captivity. One study demonstrated that the use of frozen food to reduce parasite and microbe load, use of bark instead of *Sphagnum* as cage substrate to retain moisture and reduce the incidence of blister disease, pasty diet containing thawed snakes for force feeding, and the administration of pilocarpine before milking could increase survival rates and venom yields in *Micrurus corallinus* [10]. The similarity in the venom composition profile of captive and recently caught snakes, and within captive individuals over time, has also been demonstrated elsewhere [11–13]. Researchers have also suggested that although intraspecific variability might exist between individual captive and wild type snakes, their venom pools are similar and can be used for antivenom production [14]. However, studies with *Naja atra* suggest otherwise [15]. The authors, using proteomics and transcriptomics, found that captivity can vigorously affect the venom composition between wild and captive snakes. The size, sex, and age also influence venom production [16]. Larger snakes and adult females of *Bothrops leucurus* have been found to produce more venom than small snakes and adult males, respectively. Also, the older animals may not be suitable for antivenom production due to

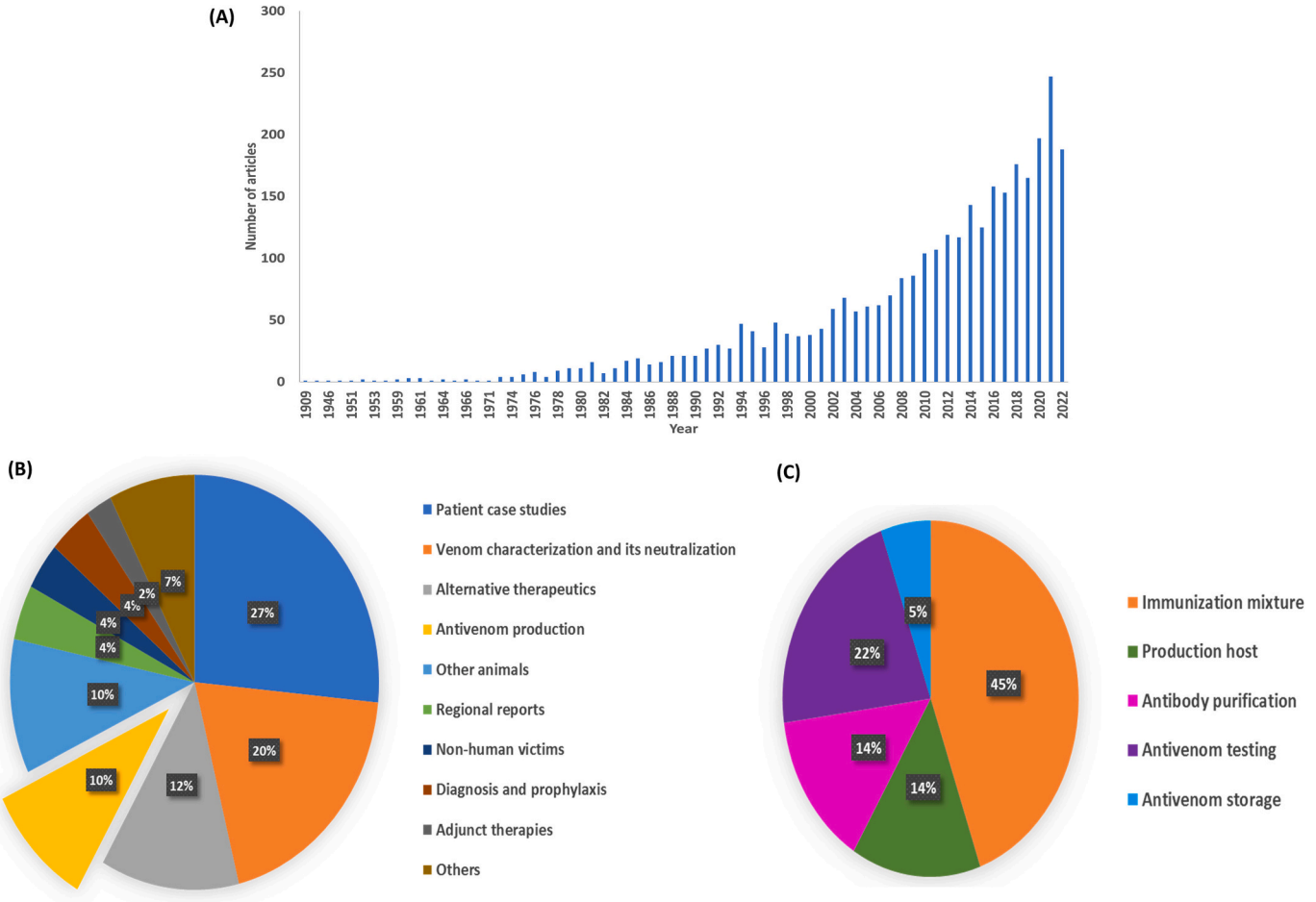

Fig. 1. Number of publications reported on antivenom. Data was collected from 'Scopus' using 'antivenom' in the 'article title, abstract, and keywords' field and the total number of research articles published each year (A). Number of research articles published from 2018 to 2022 classified according to the theme of the studies (B). Classification of the research articles published from 2018 to 2022 (yellow slice in panel B) describing advances in antivenom production (C).

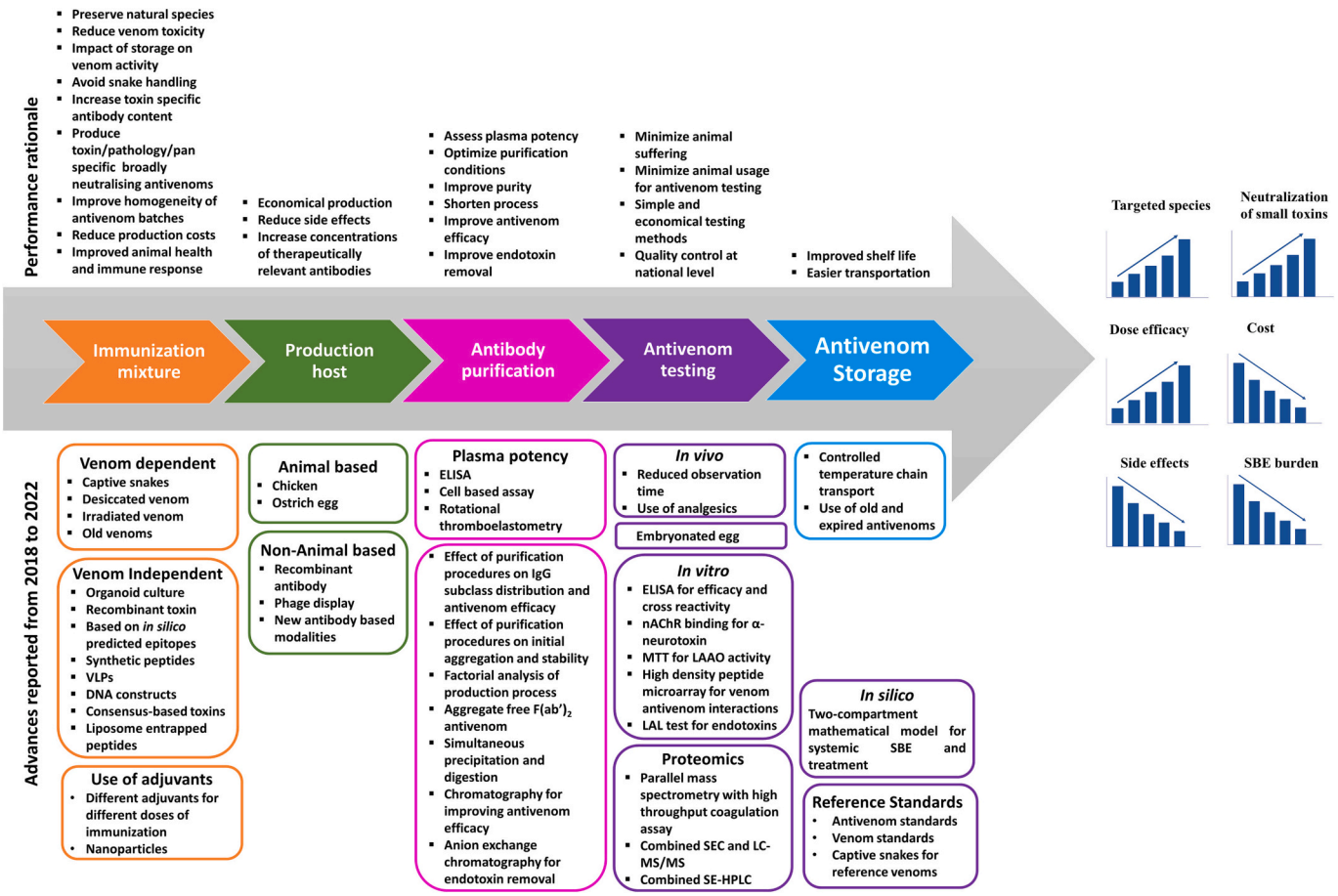

Fig. 2. Antivenom production process and recent advances. Antivenom manufacturing is a multistep process consisting of the preparation of immunization mixture, its injection in a production host, purification of the antivenom antibodies, quality testing, and the storage of the final therapeutic product. Advancements reported for each step from 2018 to 2022 and their performance rationale are summarized.

their reduced toxic activities.

The collected venom is used for antivenom production or is desiccated and stored. However, the latter may not be suitable for antivenom generation as storage can adversely impact venom toxicity and lethality. A comparative analysis of the properties of desiccated Bothrops venom collected in Ecuador from 2001 to 2016 revealed that although the protein content, PLA2 activity, thrombin-like activity, and edematogenic activities of different venoms were similar, venom lethality, hemorrhagic, myotoxic, and proteolytic activities decline upon storage [17]. On the contrary, another study found that the composition, activity, and immunogenicity of Bothrops venoms are stable up to 54 years of storage [18]. Interestingly, the authors also suggested that the drying method might influence the venom stability. Nevertheless, these contradicting studies suggest that the activities of old venoms may be thoroughly assessed before use for immunization.

Mechanical stimulation of venom glands can lead to significant tissue damage, such as fibrosis, necrosis, and edema, depending on the snake species and the frequency of stimulations [19]. Further, repeated venom extractions may affect venom composition and toxicity as well. To minimize tissue damage, the establishment of rotation groups has been suggested. Also, to facilitate venom production without encountering snakes, 3D organoid cultures of snake venom glands, from late-stage embryos to deceased adult animals, have been proposed for at least ten species [20]. Organoids can be cultured for up to two years and can be cryopreserved, allowing for the preservation of valuable cells and the expansion of organoids as needed. Besides enabling large-scale venom manufacturing without requiring the handling of snakes, the method also allows the manipulation and generation of optimized toxins. However, it must be noted that organoids are not an exact replica of venom glands, as they are only composed of epithelial cell types without neighboring muscle and nerve cells.

2.2. Generation of venom pool

Broadly specific antibodies can be elicited by increasing the diversity of toxins in the immunization mixture, for example, using multiple venoms [21]. This approach has been demonstrated to expand the neutralization potential of PoliVal-ICP, the Central American antivenom produced against venoms of *Bothrops asper*, *Crotalus simus,* and *Lachesis stenophrys*. The addition of *Crotalus durissus pifanorum* venom in the immunization mixture expanded the neutralization potential of PoliVal-ICP. It also improved the antivenom potency against *Crotalus simus* without reducing it for *Bothrops asper* and *Lachesis stenophrys* [21]. However, a mere increase in the number of venoms used for immunization may not improve antivenom efficacy [22]. On the contrary, it may reduce the proportion of antibodies against individual venoms. For instance, when the mixtures of 7 and 12 hemotoxic venoms were used for immunizing sheep, the antivenom generated with 7 venoms showed higher efficacy than the other antivenom against 12 venoms and also depicted cross-reactivity. In contrast, antivenom against 12 venoms displayed higher intensity of immunological cross-reactivity and better recognition of the low molecular weight toxins. One study suggested that the best cross-reactive antivenom-producing venoms may be selected by first developing monospecific sera against venoms from distinct species and then evaluating their cross-reactivity with homologous and heterologous venoms [23]. These venoms can then be used to

create a pan specific antivenom. Alternatively, the cross reactive antivenoms can be mixed, as per their neutralization titers, to generate a broadly reactive antivenom [24]. Although cross-reactivity studies offer a promising approach to develop broadly specific antivenoms, it must also be noted that antivenom cross-reactivities are not bidirectional. For example, the anti-*Micrurus dumerilii* antivenom can neutralize *Micrurus isozonus* venom, but not *vice-versa* [24].

To minimize intraspecific venom variability, the use of venom pools from different age and sex has been recommended. However, in cases where procurement of such variety of venoms is difficult, the use of 'average venom' has also been suggested [25]. The authors profiled venoms from 30 animals from 3 different geographical conditions using high-performance liquid chromatography (HPLC) and selected one venom containing all the HPLC fractions of *C. simus* venom for antivenom production. The corresponding antivenom neutralized all the individual venoms despite intraspecific variabilities. Interestingly, the authors also found that the animal sex did not contribute to venom variation of *C. simus* and is insignificant for consideration during antivenom production. These studies suggest that individual case by case analysis is warranted for identifying venoms and species characteristics to be used for antivenom production.

2.3. Venom detoxification

To boost the host immune response and generate antivenoms with improved potencies, venom detoxification using gamma radiation and the use of nanoparticle adjuvants have been suggested [26]. Radiation treatment can reduce venom toxicity without affecting its antigenicity. Calcium phosphate nanoparticle (CPN) can be more potent than alum and induce high titer antibodies without eliciting inflammation. In mice lethality assays, the mortality of mice administered with antivenom against irradiated CPN-loaded venom was 20 %, compared to 60 % mortality with antivenom against native venom and Freund's adjuvant, when administered immediately after venom injection. Interestingly, without adjuvants as well, irradiation can improve antivenom efficacy, and antivenoms generated with irradiated venoms have higher titer and neutralization potential than untreated crude venom-based immunogens [27]. Irradiation is also an effective detoxification strategy for venom mixtures [28]. However, it must be noted that the physical state of venom is also important. Although irradiation of lyophilized and frozen venoms does not alter venom toxicity up to 100 kGy and 40 kGy respectively, liquid venoms may be lose their toxicity by >80 % upon irradiation with doses as low as 3.5 kGy. Like crude venoms, purified venom toxins can also be irradiated to detoxify them and generate antivenoms with improved efficacies [27].

2.4. Toxin-specific antivenom and venom-independent immunogens

In a given antivenom, toxin-targeting antibodies can be as low as 5 %. The venom of a given snake species may be composed of over a hundred genes and multiple toxin families. However, only a selected few are venom specific and form the venom's core effector toxins [29]. These can be identified using genomics and next-generation sequencing technologies. Identification of venom-specific toxins would aid in developing recombinant venom substitutes for immunization and generating safe and effective antivenoms. Research could thus also be aimed at producing venom-independent immunogens such as recombinant toxins, synthetic peptides, and DNA strings [30]. Besides bypassing the need for snake management, such immunogens can be used to generate toxin-specific antivenoms. Additionally, integrated multi-omics studies would lead to better understanding of venom toxins and improve antivenom production processes [31].

2.4.1. Protein-based immunogens

The venom toxins can be separated using techniques such as HPLC, and specific toxins can then be used as immunogens for antivenom production [32,33]. The corresponding antibodies, besides neutralizing the respective toxins, can be combined to neutralize the whole venom. These toxin-specific antibodies can also be combined with antivenom against crude venom to improve its neutralization ability. Further, the anti-toxins may also show cross-reactivity against other members of the toxin family in the venom, in some cases higher than the antivenom generated with crude venom. Thus, antivenoms with enhanced therapeutic efficacies can be generated by eliminating or reducing the medically irrelevant toxins in the immunizing mixture and using relevant toxins from different species.

The other method of producing protein immunogens is using recombinant technologies, which completely eliminate the need for snake management and venom production for antivenom manufacturing [34–36]. The commonly used *E. coli* system can be used to express the recombinant toxins, or venom toxins fused to immunogenic carrier protein [34,35,37]. However, prokaryotic systems lack the enzymes required for post-translational modifications or disulfide bonding which are frequent in snake toxins. Thus, to generate native-like forms in *E. coli*, venom toxin can be fused with a disulfide isomerase to aid in appropriate disulfide bond arrangement and correct protein folding [35]. Another method of producing recombinant toxins is using mammalian systems such as the human embryonic kidney cell line HEK293 [36]. These cells have been reported to produce purified toxins with a yield of 0.7 g/L.

Toxin neutralization can also be achieved by targeting specific domains instead of whole toxins. For example, antibodies against disintegrins can also be used to neutralize snake venom metalloproteinases (SVMPs) [38]. Disintegrins are important venom components that can also be produced upon proteolytic processing of PII/PIII SVMPs. Anti-disintegrin antibodies can thus be used to neutralize SVMPs, the proteolytic activity of venoms, or crude venoms such as *C. atrox,* whose major constituents are SVMPs.

2.4.2. Virus-like particles (VLPs)

Owing to the poor immunogenicity and high cost of chemical synthesis, peptides can be fused to virus-like particles (VLPs) [39]. VLPs are highly immunogenic empty virus particles without nucleic acid and are approved antigen carriers for vaccine therapy in clinics [40]. Epitopes pertaining to the predicted conserved regions of venom toxins can be displayed on VLPs, which can further be used for immunization to generate cross-reactive immunoglobulins and for targeting low molecular weight toxins. When working with the epitopes, it must also be noted that the sequence is important since epitopes differing by only a single amino acid may exhibit different abilities to recognize venom components. For example, the presence of proline in the sequence may be crucial for certain venom recognition.

2.4.3. Low molecular weight toxins

When selecting venom toxins for immunization, large, high molecular weight toxins are usually considered. The small, low molecular weight toxins, such as three-finger toxins (3FTxs), despite their high toxicity, are generally neglected for their low immunogenicity. However, recent studies suggest that intermediate to high-affinity T4 immunogenic epitopes are present on these molecules [41]. The presence of CD4 T cell epitopes indicates the potential of these toxins to stimulate specific B cells for antibody production. Indeed, neutralizing antibodies can be produced against 3FTx-containing venoms, and broadly neutralizing antivenoms can also be generated [35,39,42,43]. The antivenom produced using 12 neurotoxic toxins/venom from six species can neutralize 36 venoms belonging to species from 4 continents and spanning 10 genera. These studies challenge the general belief that small molecular weight toxins have low immunogenicity and contribute to the low potency of antivenoms. Also, they demonstrate that potent antivenoms against these toxins can be produced with appropriate adjuvants and immunization methods without physical and chemical modifications.

2.5. Pathology-specific antivenoms

To address the challenge of producing broadly reactive antivenoms, the niche of pathology-specific antivenoms can also be explored [36]. For example, immunization with snake venom serine proteases (SVSPs) can produce an antivenom that inhibits the fibrinogenolytic activities of geographically diverse snakes. Since the antibodies will be toxin specific, their binding to SVSPs would be greater than antivenom generated against crude venom. Another way to develop pathology-specific antivenoms is by pooling venoms from diverse but toxicologically similar snakes [22]. For example, venoms from diverse hemotoxic snakes can be pooled to develop therapy against snake hemotoxicity.

2.6. Synthetic immunogenic epitopes

The immunogenic epitopes in venom toxins can also be directly targeted. For example, Melo et al. used sets of overlapping 15 mer peptides to cover the complete primary sequence of both crotamine and crotoxin. The antigenic regions were then identified using anti-*C.d. terrificus* venom horse IgGs [44]. For all the identified epitopes, the corresponding synthetic peptides were entrapped in liposomes for antivenom production in rabbits. The antibodies successfully neutralized the lethality of *C.d. terrificus* venom with up to 50 % mice survival 48 h post venom injection. This selective epitope approach can be helpful for producing antivenoms against venoms of high toxicity and those containing immunosuppressants which lead to the production of low titer antibodies. These epitopes can also be combined with crude venoms for immunization. For example, a cross reactive neutralizing rabbit antivenom was generated using crude venom of *M. frontalis* for priming and cocktail of validated synthetic B cell epitopes of 3FTx and PLA2 from *M. corallinus* for booster [45].

2.7. DNA-based immunogen

Another alternative to using venom(s) as an immunizing mixture is immunization using DNA constructs encoding venom toxins or epitopes. DNA synthesis is cheaper than peptides and recombinant proteins. A computational pipeline to predict the most relevant epitopes for antivenom production using known toxin sequences of medically relevant snakes has been recently developed [46]. Using bioinformatics analysis, such as sequence conservation and protein toxicity scores, the most relevant conserved epitopes can be selected and incorporated into a DNA construct for immunization. Although the experimental validation of the construct is pending, theoretically, the proposed construct can neutralize venom from several species independent of their geographical location. An advantage of using *in silico* tools is that sequence similarities between species can be analyzed [46]. While epitopes conserved across multiple snake species suggest possible cross-reactivity, sequences similar to human proteins may need to be modified to prevent cross-recognition [44]. A cross-reactive broadly neutralizing antibody pool thus can be generated using interspecies conserved toxins. Another study used the sequences of elapid venoms from Asia, Africa, America, and Oceania, and generated antivenom against recombinantly produced consensus short chain α-neurotoxin [47]. The resultant equine antivenom exhibited broad cross-reactivity and neutralized lethality of native toxin and whole venoms from diverse genera of neurotoxic elapids. The authors proposed that such consensus-based toxins can act as universal immunogens for the production of broadly specific antivenoms.

To develop vaccine based antivenom against the neurotoxic activity of *B. candidus* venom, one study predicted the potential immunogenic epitopes on candoxin bioinformatically [48]. The authors modelled the epitopes and analyzed their interactions with murine and human MHC-II. After excluding the allergenic properties and analyzing the *in vivo* stability using physicochemical property analyses and molecular dynamics simulations, one epitope was predicted as a potential candidate for vaccine based antivenom. Potentially immunogenic epitopes have also been predicted on PLA2 from *Bungarus candidus* using docking studies [49].

These advancements can be particularly significant for species with difficult venom production owing to difficult maintenance in captivity and low venom production [33]. By minimizing the number of antigens injected, immunization with purified toxins improves the health and lifespan of production hosts, thus allowing the generation of more effective antivenoms at reduced costs [27]. Further, the use of toxin-specific antibodies would enhance the dose efficacy of antivenoms. Researchers showed that a cocktail of test monoclonal antibodies (mAbs) exhibited higher *in vitro* inhibitory potential than commercial antivenoms [43]. Thus, toxin-specific antivenoms can also reduce the amount of antivenom required to be administered and hence decrease side effects. However, toxin or pathology-specific antivenoms may not be effective against venoms lacking the toxins used in immunization mixture or whose lethality depends on the synergistic action of multiple toxins [36,42].

Adjuvants are often added to immunizing venom to boost the immune system's antibody response. Adjuvant development for antivenom synthesis, however, is a topic with limited research. The search for articles containing 'antivenom' returned only a few reports that investigated the effect of adjuvants on antivenom production [50–52]. In one study, the authors suggested that the use of different adjuvants for different doses of immunization – Freund or Montanide for first immunizations, and Emulsigen D or similar adjuvants for later doses-might result in satisfactory antivenom production in horses without deleterious effects [51]. Another group demonstrated the potential of chitosan nanoparticles as immunoadjuvants for antivenom production [50,52]. The chitosan nanoparticles were loaded with *Bothrops jararaca*, *Bothrops erythromelas*, or *Crotalus durissus cascavella* venom using the ionic gelation method. With a slow protein release profile, the biodegradable and biocompatible nanoparticles generated equivalent or higher antibody titer, than the conventional aluminum hydroxide, demonstrating the ability of these nanoparticles in the production of polyclonal serum. Adjuvant SBA-15 has also been shown to be effective in raising venom neutralizing antibodies in mice [32].

3. Production host

Generally, the venoms are injected into large animals such as horses and sheep for antivenom production. However, hyperimmunization with large amounts of venom can cause substantial adverse reactions, pain, and suffering to the animal. To this end, the concept of low dose, low volume, multi-immunization has been recommended [53]. This involves injection of small volume of immunogen, containing low venom doses, at multiple sites. For example, when tested with *Vipera ammodytes* venom, this protocol increased the equine antivenom titers to up to 8 folds while considerably reducing animal suffering, with reduced requirements for venom, snakes, and production costs [54]. The use of other animals, such as chickens, ostriches, and camels, for antivenom production, has also been proposed [55–57]. While camelid antibodies offer high thermal stability, using chickens and ostriches offer non-invasive and economical method of antivenom production. Recent studies advocating the use of alternative production hosts have been discussed below.

3.1. Chickens

The scope of chicken IgY antibodies for venom neutralization and cross-reactivity has been confirmed in several studies [58,59]. For instance, 90 days post-immunization and administration of booster doses, immunization of chickens with snake venoms has been reported to yield a high IgY titer of even 1:64000 [58]. A comparison of the efficacy of chicken IgY, produced against *Daboia russelii* and *Echis carinatus* snake venoms, with corresponding IgG antivenom antibodies revealed

that in sandwich ELISA, IgY detected up to 0.1 ng venom. In contrast, IgG could detect venoms at 100 ng. The IgY also displayed cross-reactivity to other venoms and was needed in similar amounts as rabbit IgG for *in vivo* neutralization of venom lethality. However, it was less stable than the IgG. In another study, the authors demonstrated the ability of IgY to recognize low (<20 kDa), medium (20–40 kDa), and high (>40 kDa) molecular weights venom toxins [60]. The IgY antivenoms offer several advantages over conventional equine IgGs, including cheaper production. As per the estimates, 63 chickens produce as many antibodies as a horse per year. Additionally, IgY antibodies do not activate the complement system. Hence the availability of IgY-based antivenoms might allow physicians to administer larger doses to patients. However, additional pre-clinical studies are needed to fully validate the therapeutic relevance of chicken IgY antivenoms.

3.2. Ostriches

Another approach to producing IgY antivenom in large amounts is using ostriches [57]. Immunization of ostriches with venom pool can produce high titer-neutralizing antibodies. Notably, the titer does not decline significantly when eggs are kept at 5 °C during a year. Ostriches can thus be an economical production host for antivenom production as a single egg can produce about 2-4 g IgY, and approximately 400 g IgY can be generated annually from a single animal. Further, the monthly maintenance cost of one ostrich is approximately USD 146, which is far cheaper than the USD 100 required maintaining one horse per day. Using ostrich eggs for antivenom production is a non-invasive and economical method for antivenom production. However, additional studies are required to explore its potential in the future.

3.3. Recombinant antibodies

Besides the above developments, there has been growing interest in the production of recombinant antibodies, the use of phage display technologies, and the development of single chain variable fragments (scFv), including those based on chicken IgY [59,61–66].

A mixture of recombinantly produced mAbs can be used to target the diverse repertoire of venom toxins [67–69]. However, since the number of mAbs that can be mixed is limited, broadly neutralizing mAbs are required to target the diverse repertoire of venom toxins [68]. Further, if such mAbs show cross-reactivity against venom toxins from different species, a polyvalent oligoclonal antibody mixture can be generated. Such polyvalent and broadly neutralizing mAbs can be identified using cross panning strategy and phage display technology [70]. Phage display can also be used to identify a single antibody capable of neutralizing whole venom [65]. For example, a single human monoclonal IgG against α-cobratoxin capable of neutralizing whole snake venom of *N. kaouthia in vivo* has been identified using phage display and affinity maturation. Another study showed that an oligoclonal mixture of three recombinantly produced fully human IgGs, when administered through the intracerebroventricular route, can neutralize the dendrotoxin mediated neurotoxicity of the whole venom of *Dendroaspis polylepis* in rodents [71]. Not only the mAbs, the Fab fragments can also be expressed recombinantly in bacterium, purified, and used as antivenom [72]. This approach has been shown to neutralize *Echis carinatus* venom with a potency of 7 LD_{50}/ml.

Although these results are promising, the limited antibody combinations may not work against small toxins or venoms whose toxicity depends on synergistic action of several toxins. In this regard, plant biofactories can be considered for production of recombinant polyclonal antibodies [73]. Since plant viruses exhibit superinfection exclusion, which prevents reinfection of cells with a second related virus, several different antibody producing mosaics can be generated in plant leaves in a reproducible manner. This approach allows production of hundreds of idiotypes simultaneously. The recombinant antivenom so produced had venom binding profile similar to previously reported equine anti-Bothrops antivenom and has been shown to neutralize *in vivo* toxicity and lethality of *Bothrops asper* venom. Plant-based production systems offer several advantages such as easy scalability, low production costs, and enhanced safety profile. However, additional studies are required to fully understand the pros and cons of plant-based antivenoms before they are to be accepted for clinical use.

While animal-derived antivenoms may cost USD 13- $1120 per treatment, recombinantly produced monovalent and polyvalent antivenoms can be produced at USD 20-$225 and USD 48-$1354 per treatment, respectively [74]. However, before recombinant antivenoms enter clinics in the future, appropriate guidelines, and their classification into blood products or biotherapeutics need to be mandated [65].

4. Antibody purification

Post immunization, the antibody response in the animals is monitored throughout the immunization period. When sufficient antibody titer is reached, the animal is bled, and the plasma is fractionated to purify the antibodies.

4.1. Estimation of plasma potency

Enzyme linked immunosorbent assay (ELISA) can be used to assess the potency of hyperimmunized equine plasma [75]. Using only 10 ng venom antigen per well and 1:20000 diluted plasma, a comparison of antibody titers against major toxins of *N. atra* venom revealed that the titer against venom neurotoxins was proportional to antivenom potency. The assay can use crude plasma samples without IgG purification and has high sensitivity and specificity of 96.88 % and 89.47 %, respectively. The ELISA test can also be used to monitor antibody response in immunized horses, determine the time point and select animals for bleeding, and also plasma samples for processing [54,75,76]. Accurate estimate of the purification process efficiency requires precise IgG measurement in plasma. Results, however, can be affected by variations in sample and standard IgG composition. Thus, sample-specific references should be utilized to measure plasma IgG precisely and accurately in immunoassays [77].

It must also be noted that ELISA is a binding assay which detects the interactions between venom and antivenom components. The results may thus not always correlate with the functional neutralizing ability of antivenoms especially in cases where venom toxicity is dependent on toxins with low immunogenicity. In cases where the signatures of systemic envenomation are defined, the viability of venom treated cell line derived from an affected organ can be used to estimate antivenom potency [78]. For example, systemic envenomation by *Bothrops jararaca* is known to affect kidneys. Thus, the cell viability of kidney derived Vero cell line in presence of *B. jararaca* venom and antisera can be used to estimate the potency of the latter. The IC_{50} can then be used to establish a cut off value to differentiate between the high and low potency sera.

4.2. Antibody purification

The antibodies are purified from the animal plasma in a multi-step process [5]. It involves antibody precipitation followed by digestion and chromatographic or filtration-based purification. Digestion is performed to produce Fab and $F(ab')_2$ based antivenoms with further improved safety due to the removal of the Fc region, and neutralization than IgG-based antivenoms [67].

4.2.1. Precipitation, digestion, and chromatographic purification

Traditionally, ammonium sulfate or caprylic acid are used to precipitate the antibodies or non-IgG content from the plasma of hyperimmunized animals, respectively. The precipitation efficiency of caprylic acid has been observed to be more than ammonium sulfate [80]. For IgG extraction, ion exchange and affinity chromatography have also been suggested. However, a recent study reported that

chromatographic purification might lead to a substantial loss of IgG(T), the most important IgG subclass for toxin neutralization, and reduce neutralization activity by >50 % [81]. The authors found that precipitation, unlike chromatographic methods, does not affect IgG subclass distribution, and caprylic acid gives >90 % purity in a single step with low aggregation while retaining the plasma-specific activity. Anion exchange chromatography minimizes aggregation but must be performed at low pH else IgG(T) loss can happen. Thus, the effect of the purification procedure on IgG subclass distribution and antibody activity must also be considered during protocol designing. The choice of purification process also affects the stability of the final antivenom product [82]. The aggregation propensity of antivenoms was found to be dependent on the initial content of aggregates, which in turn is determined by the purification method. Antivenoms prepared using mild purification methods such as anion exchange chromatography and caprylic acid precipitation, result in low levels of initial aggregation and a more stable product. The selection of appropriate purification methods is thus necessary for manufacturing antivenoms with long shelf lives.

Since antivenom purification is a long and sophisticated process, the most relevant conditions for different steps must be identified for efficient production. A factorial analysis of antivenom production processes suggests that maximum conversion of equine IgG to $F(ab')_2$ with minimal aggregation can be achieved with a 60 min incubation of 1:3 saline diluted plasma at 37 °C, pH 3.2 and 1:15 pepsin to substrate ratio [83]. The quality depends inversely on the albumin to protein ratio in the original plasma and directly on the ammonium sulfate concentration used for purification. However, high concentrations of the latter can also decrease the recovery. Compromise between monomer recovery and quality can be obtained at 14 g/dl concentration at 56 °C and at 16 g/dl concentration at 30 °C. The optimized conditions can give a 42 % yield with 2.5 % protein aggregates using traditional methods without significantly increasing the cost.

The production of $F(ab')_2$ antivenoms that are 100 % pure and aggregation free might also be possible by maintaining the product in solution continuously during the purifying procedure, including during the phases of precipitation, digestion, and chromatography [84]. This would preserve the antibody conformation and a homogenous, aggregate-free, and pepsin-free product can be obtained with a yield of over 75 % by combining optimized conditions, such as precipitation with 2 % caprylic acid, pepsin digestion at pH 3.2 for 1.5 h at an enzyme substrate ratio of 4:300, and sequential purification *via* diafiltration and chromatography.

To shorten the purification process, antibody precipitation and digestion can also be performed simultaneously [85]. For preparing equine $F(ab')_2$ antivenom, IgG precipitation with caprylic acid and pepsin digestion with 1:30 pepsin to substrate ratio at pH 3.2 at 21 °C for 2 h can be used. This can then be followed by diafiltration and chromatography for contaminant removal. The possibilities of structural and conformational changes during the procedure can be minimized by keeping the $F(ab')_2$ in solution in each phase. This approach can yield aggregate and pepsin-free product with just three processing steps, with an overall yield of 74 % and protective efficacy comparable to plasma IgG. However, the proposed method is host specific, and optimizations are required for antivenoms produced in different production hosts.

Antivenom purification procedures can directly impact antivenom efficacy [86]. A comparison of chromatographically purified *versus* commercial antivenoms revealed that chromatographic purification, besides increasing the purity and/or fraction of toxin-binding antibodies can also enhance venom binding and the *in vivo* neutralization potency of the antivenoms by about 3 to 4.5 times. The addition of chromatographic purification can thus enhance antivenom dose efficacy and reduce impurities and side effects.

4.2.2. Endotoxin removal

Endotoxins can be immunogenic. Hence, ultrafiltration and chromatography-based approaches have been described for their removal. Researchers compared these techniques to determine the method with the highest efficiency and antivenom protein recovery [87]. The effects of different buffer pH and ionic strengths have also been investigated. The highest protein recovery of 91.2 % could be attained with affinity resin chromatography at acidic pH. Anion exchange chromatography with 0.3 M NaCl at pH 7.5 recovered 74.42 % protein, while ultrafiltration was ineffective for removal of albumin and low molecular weight contaminants. The study demonstrated that ultrafiltration may not be suitable for endotoxin removal from serum antibodies and the importance of pH in producing endotoxin-free and sterile antivenoms.

5. Antivenom testing

Once the antibodies are purified, they need to be tested for their therapeutic potential. For instance, in Africa, where sub-optimal antivenoms were used following the cessation of the FAV-Afrique, an increase in mortality was observed [88]. Antivenom testing before releasing them into the market is thus crucial for ensuring antivenom efficacy. The gold standard test for assessing antivenoms' efficacy is its ability to neutralize venom lethality in animal models [89]. However, the WHO recommends the implementation of the 3R's (reduction, refinement, and replacement) to minimize animal usage for antivenom testing [90,91]. Several modifications in the testing procedure have thus been suggested, and the recent ones are discussed below.

5.1. In vivo assays

Currently, as per the protocol recommended by the WHO, venom-induced animal deaths are recorded for 24 h or 48 h, and the obtained results are used to calculate antivenom potency (ED_{50}). However, a recent study suggests that LD_{50} and ED_{50} values calculated at 6 h endpoint correlate with 24 h and 48 h time points. Hence, 6 h observation times may be used to minimize animal suffering [92]. Similarly, to reduce the pain and suffering during antivenom efficacy testing, the mice can be pretreated with analgesics such as morphine and tramadol which do not interfere with venom toxicity [93]. The use of alternative animals has also been suggested. For example, *Artemia salina* can be used as a potential surrogate model for analyzing dermonecrosis, a clinical complication of envenoming by snakes such as cobras [94,95]. The *A. salina* bioassay at 24 h exhibits a good correlation with the dermonecrosis bioassay performed in mice at 72 h and offers a simple, rapid, and economical alternative to similar assays in mice for evaluating venom toxicity and neutralization by antivenoms.

5.2. Embryonated egg model (EEM)

Another way to avoid using mice as models for antivenom testing is using EEM [96]. In this, the venom is injected into the egg albumin of the six days old embryonated chicken eggs, and the viability is analyzed by candling. The method is compatible with multiple snake venoms, including those containing neurotoxic, cardiotoxic, myotoxic, and pore-forming toxins. The embryos are euthanized before 50 % gestation, a time when the embryo remains insensate, *i.e.*, the nervous system is not fully developed to experience pain. Also, during this time, the embryos are not called animals and hence are not subject to welfare legislation (at least in Australia).

5.3. In vitro assays

The in-process evaluation of antivenom efficacy can also be achieved using *in vitro* assays [89]. For example, the toxin epitopes can be hammered into synthetic immunoreactive peptides that compete for binding with antivenom antibodies. Incubation of such peptides with venom antivenom mixtures and their injection into the animals can be used to identify the neutralizing antibodies in the antivenom [97].

Further, the results of competition assay combined with peptide antibody titer studies with ELISA can be translated into the neutralizing potency of antivenoms. Thus, using specific peptides to different venom components, the assay can be used to estimate antivenom efficacy against individual toxins and to determine cross-reactivity.

An *in vitro* nicotinic acetylcholine receptor (nAChR) binding assay can be used as a substitute for mice lethality tests for α-neurotoxin dominated neurotoxic venoms [98,99]. The assay is based on the binding of α-neurotoxin in snake venoms to the soluble nAChR, an abundant toxin in the venoms of many elapid snakes. The method has good correlation with the *in vivo* determined LD_{50} of 20 elapid venoms (R^2 = 0.852) and ED_{50} values of the pan-specific antivenom (R^2 = 0.689). The assay is simpler, faster, more economical, and reproducible than the standard mouse lethality tests. Also, it can be used as an alternative to isolated nerve-muscle preparations, which are technically difficult and time-consuming for routine investigations.

An *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for characterizing L-amino acid oxidase (LAAO) activity in venoms has also been developed [100]. Using *Vipera ammodytes* venom, the researchers found that the LAAO in venom reduced MTT in the absence of cells in a concentration-dependent manner. Further, the ability of LAAO to act in monomeric form was confirmed along with good correlation (R = 82.57 %) with the standard antivenom potency tests.

Rotational thromboelastometry can also be used to assess how well anti-sera inhibit venom activity on the coagulation response in recalcified chicken plasma samples [79]. The authors used this assay to determine the relative potency of four batches of anti-crotalic serum in inhibiting the anticoagulant effects of crotoxin in chicken plasma samples that had been simultaneously treated with a conventional activator of coagulation. The assay had almost hundred times more sensitivity than conventional methods and related well with the *in vivo* inhibition of venom lethality (r = 0.94).

Another way of antivenom testing is based on determining venom-antivenom interactions. One such method is high-density peptide microarray (hdpm), but its analysis needs expert understanding. To ease this data analysis, a free online application, 'snake toxin and antivenom binding (STAB) profiles', has been developed [101]. The tool has been tested with the largest venom antivenom dataset constructed using a 16-mer peptide library from protein sequences of all the African snake toxins available in Uniprot and 8 African commercial antivenoms and conducting hdpm assays. It can also be used to differentiate the interacting potential of different antivenoms with specific toxins of interest and understand antivenom cross-reactivity and para-specificity.

To estimate the levels of $F(ab')_2$ antivenom against *Daboia russelii siamensis* in human serum, a ligand binding assay has been developed and used for phase I clinical studies in healthy volunteers [102]. The method had an accuracy and precision of ±20 %, was validated according to the regulatory guidelines, and is suitable for clinical pharmacokinetic research. The method was free from the hook effect, interference from exogenous substances, hemolysis, and hyperlipidemia, with the mean correlation coefficient R^2 of 0.9981.

The endotoxins in antivenom are tested *via* the Limulus amebocyte lysate (LAL) test. To enhance method specificity and reduce false positives due to interfering agents, 10 min heating at 70–80 °C followed by rehydration with specific buffer solution has been recommended [103].

5.4. Proteomics

Researchers have integrated parallel mass spectrometry with high throughput coagulation assay to assess the coagulopathic potential of nano-fractionated venom toxins and the ability of antivenoms to neutralize each of them [104]. The method was also suitable for testing antivenom cross-reactivity against closely related species. This approach can act as a complementary tool for antivenomics analysis. However, nano-fractionation may disrupt the native state of toxins, and usually co-eluting toxins are obtained, which may distort the bioactivity chromatograms. Nevertheless, the method allows analysis of antivenom potential to neutralize separated venom toxins instead of crude venoms.

Liquid chromatography-tandem mass spectroscopy (LC-MS/MS) can also be combined with size exclusion chromatography (SEC) in an integrated proteomic method to analyze the physiochemical purity of antivenoms [105]. Antivenom composition can be determined based on the molecular masses obtained with SEC and MS-based protein identifications. Besides the IgG chains, this approach can be used to detect large proteins such as alpha-2-macroglobulin, IgG aggregates, and impurities such as albumin. The proposed MS-based analysis is suitable for qualitative and quantitative assessment of antivenom and hence can be useful for pre-clinical assessments of antivenom purity. Although the method is expensive and sophisticated and might not be suitable for routine use, it may be useful as a complementary protocol for profiling antivenoms with apparent impurities. The use of SE-HPLC has also been suggested for assessing the binding of commercial antivenoms to venom toxins and quantifying the venom-antivenom complexes [106]. Based on SE-HPLC binding assay and the *in vitro* functional enzymatic assays, the authors computed an efficacy score to predict ED_{50} and quantitate the efficacy of antivenoms. The approach was also able capture the geographical variation in venoms of same species.

5.5. In silico studies

Besides the *in vitro* tests, *in silico* tools can also be used to predict the neutralization potential of antivenoms [107]. For example, the ability of antivenoms to treat SBE can be tested using a two-compartment mathematical model for systemic SBE and treatment. The distribution and elimination dynamics of different antibody modalities, namely Fab, $F(ab')_2$, IgG, ScFv, and nanobody, can be predicted based on their molecular weights. The developed model can also be used to simulate intramuscular envenomation and its neutralization using Fab, $F(ab')_2$, and IgG antivenoms. Besides the quantitative assessments of antivenoms, the model can also be used to predict systemic toxin impact and neutralization in different parts of the body and to estimate optimal dosing.

For quality control of antivenoms at the national level, national or regional reference standards for antivenom can be established, as recommended by the WHO. Recently, the first national reference standard for antivenom in Korea has been established [108]. The antivenom may be used as standard in other regions as well that are inhabited by Gloydius species. The use of such standards would ensure quality control of manufactured antivenoms. Venom standards can also be used to estimate antivenom potential [13,109]. For example, the Brazilian crotalic reference venom is used to estimate the potency of Brazilian crotalic antivenoms [13]. In Korea, the first venom standard was established in 2004, and recently, the second national reference standard for snake venom has been established [109]. For production of these reference venoms, the use of captive snakes has also been suggested [12,13]. However, regarding the production of standard venoms, clear guidelines from regulatory authorities are required for the milking and use of animals for reference venom production.

Thus, multiple *in vitro* tests can be performed to test antivenoms, but if they are to be accepted, appropriate benchmarks for each test need to be framed [110]. Similarly, appropriate quality control procedures are mandated by the WHO at every step of the production process starting from species selection to testing of final antivenom product. Some of the major processes in this regard are listed in Table 2. For a detailed discussion about the quality control of antivenoms, the readers are referred to other reviews and the regulatory guidelines by the WHO [5,90,111–113].

6. Antivenom storage

Antivenoms are often transported in cold chains which makes their

delivery in remote regions difficult and costly. A study on the stability of antivenoms outside the recommended storage conditions suggests that under accelerated stability test conditions, the liquid antivenom can retain its protein content, composition, and potency when stored at 35 ± 5 °C for one week [114]. Although *in vivo* validation of these results is pending, this study indicates that controlled temperature chain transport of antivenoms might be feasible. In several studies, antivenoms have been shown to retain their physical, chemical, and functional properties up to 20 years beyond their expiration dates [115–117]. Results of *in vitro* assays suggest that proper antivenom storage can preserve the efficacy of liquid antivenoms as well [115]. Administration of 124 vials of lyophilized equine $F(ab')_2$ antivenoms expired for 2–60 months to 31 patients during medical emergency Lao People's Democratic Republic resulted in full recovery of all 31 patients from systemic envenoming for a median follow up of 6 days [118]. Despite their expired nature, the average 3.6 antivenom vials were required per patient having hemotoxic envenoming. This was less than the non-expired antivenoms used in other reports. Additional studies on antivenom shelf life, and preclinical assessments of expired antivenoms can thus save many lives and are additional avenues for future research in the antivenom manufacturing domain. This can be of particular significance for the countries facing antivenom shortage.

7. The BASE concept

The BASE concept is an intriguing synopsis of the ideally balanced antivenom. The abbreviation BASE refers to "broadly specific, affordable, safe, and effective" (Fig. 3). This idea incorporates features that antivenom manufacturers must strive to attain. Each component represents information used in developing strategies to produce an ideal antivenom. For instance, any antivenom must meet the "basic minimum" standards of being both safe and effective, and a product that does not meet these criteria is useless for therapeutic purposes. The least immunogenic and risk-free antivenom must also be used, or else the clinician's evaluation of the risk-benefit ratio would always govern how much is administered and whether or not to provide the antivenom. This would create an unacceptable uncertainty as far as the victim's life is concerned. It is also essential to remember that SBE is primarily a disease of the poor, therefore, the therapies must unavoidably be inexpensive. Thus, a good antivenom would be one that is effective, safe, and affordable. However, development of these antivenoms would not be commercially feasible in the already small and fragmented antivenom market if they lack broad applicability across various snake species. Therefore, it is crucial to include the BASE target in the antivenom production process to accomplish the WHO roadmap. This BASE notion ought to serve as the basis for the creation of alternative antivenoms as well.

8. Conclusion and future perspectives

Apart from the generation of new antivenoms, improvements in product quality and enhancing the production capacity are critical actions for SBE management and are the focus of the WHO 2021 roadmap for managing SBE. These goals can be achieved by overcoming the limitations of the current antivenom production processes. The venom pool variability can be eliminated with recombinant or synthetic immunogens, and the antivenom immunogenicity can be reduced with IgY, new antibody modalities, recombinant antivenoms, and optimum purification procedures. Similarly, low molecular weight toxins can also be targeted with appropriate adjuvants and immunization methods. Antivenom cross-reactivity and dose efficacy can be enhanced using diverse toxin repertoire, toxin-specific, and pathology-specific antivenoms or with the use of conserved toxins. Animal usage for antivenom testing can be reduced with *in vitro* assays and proteomics studies. Additionally, *in silico* tools are emerging for identifying immunogenic epitopes for immunogen, understanding venom-antivenom interactions, and antivenom testing. *In silico* tools are further fuelling innovation in antivenom research. Having antivenoms that can be stored for longer periods of

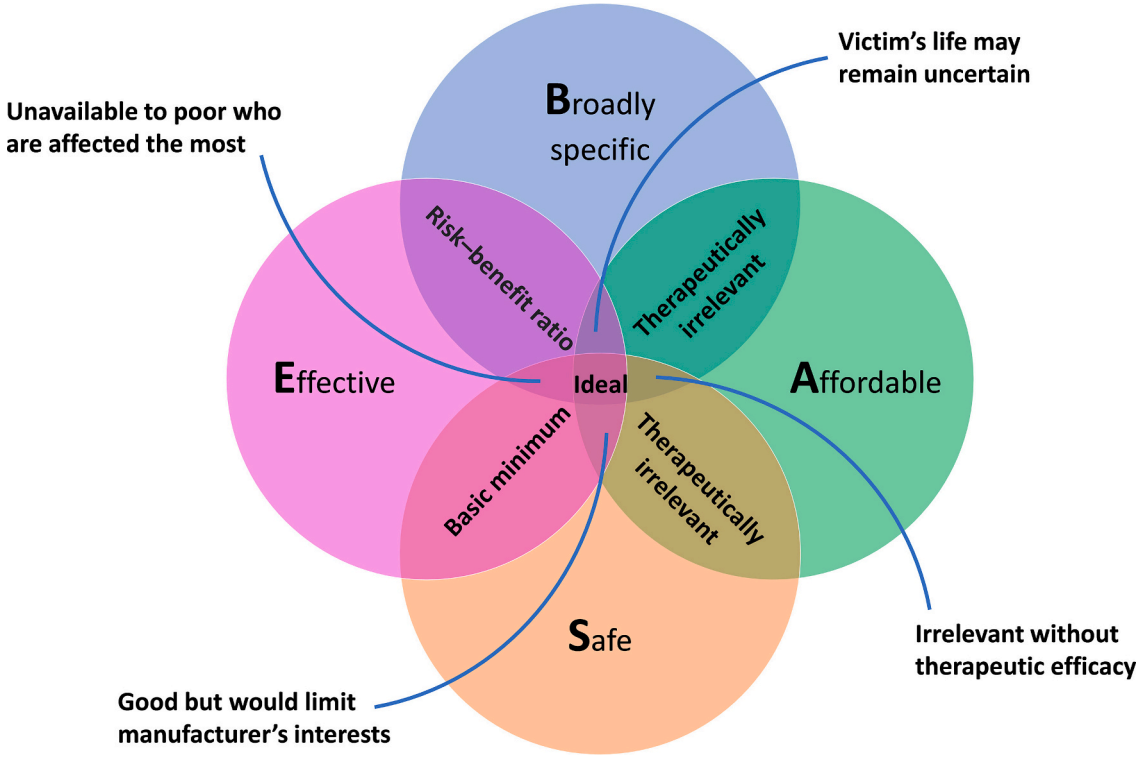

Fig. 3. The BASE objective of antivenom production. The Venn diagram represents the four objectives *i.e.*, BASE (**B**roadly specific, **A**ffordable, **S**afe, and **E**ffective) and their relevance in antivenom production. The overlapping areas represent the impact of respective parameters on antivenom therapy and market. Only the intersection of all four can lead to the generation of an ideal antivenom.

time and at higher temperatures helps ease the transportation and scarcity crisis. Although these developments are promising, several factors must be considered, and each parameter demands careful evaluation (Table 1). Finally, the antivenom production must be aimed at generating BASE antivenoms which would contribute to greater homogeneity in antivenoms across regions, reduce production cost, and stabilize the fragmented antivenom market.

The best course of action for reducing SBE burden is to concentrate on improving the current antivenoms because developing new alternatives and commercializing them is likely to take time. The aforementioned developments in antivenom manufacturing at the various stages will result in 1) improved prognosis with the availability of quality antivenoms, 2) reduced dosage and side effects, 3) and wider population coverage with economical antivenoms. However, there is still substantial room for additional studies and development. For example, integrated multi-omics and bioinformatics studies will improve our understanding of venom toxins and antivenom manufacturing technologies. Further, data from existing venomics, transcriptomic, and other multiomics studies can be compiled to map the conserved toxins and epitopes. Antisera against these toxins can be developed and tested for cross-reactivity with related species. This will expand the neutralization potential of antivenoms without detailed venomics analysis of the related species. Similarly, efforts must also be focused on *in vitro* venom production systems. Despite the possible availability of synthetic or recombinant immunogens in future, such systems might allow production of specific immunogen mixtures for targeting species with diverse toxin compositions. Likewise, more efforts must be made to minimize toxicity of the immunization mixture and generate maximum immune response with minimum amounts of venom such as with the use of appropriate adjuvants. The potential of advanced methods for immunogen delivery

Table 1
Dos and Don'ts for production of an ideal snake antivenom.

Aim	Do	Don't	Reason(s)	Reference(s)
Increase species reactivity/ develop broadly specific antivenom	Select immunogen carefully, use interspecies conserved toxins, Pool venom/toxins from multiple species	Increase venom pool irrationally	• Increase in venom pool may decrease the proportion of antibodies against each constituent venom • Immunization with diverse antigens allow generation of antibodies with diverse paratopes for interaction with toxins from homologous and heterologous venoms	[22,39,42,46]
Use desiccated venom	Confirm venom and toxicity profile are similar	Use desiccated venom directly	Venom toxicity, lethality may decline upon storage which can affect its immunogenicity	[17]
Target 3FTx and small molecule toxins	Use appropriate adjuvant and immunization method	Neglect small toxins for their low immunogenicity	Immunogenic epitopes are found on small toxins and specific antibodies can be generated against them	[33,35,39,41–43]
Increase toxin specific antibodies, decrease antivenom dosage and side effects, increase efficacy	Use only medically relevant toxins for immunization, Purify antibodies	Use whole venom for immunization, skip purification	Not all the venom components are therapeutically relevant, purification enhances quality	[27,33,35,36,38,39,43,44,86]
In vitro testing of antivenom activities	Optimize assay conditions for each venom or venom/antivenom pair	Use same conditions for different venoms and antivenoms	The assay output might depend upon the intrinsic properties of venom and antivenom, such as venom toxicity profile and antivenom composition	[75,96,100]
Produce recombinant antibody mix with broad specificity	Use broadly neutralizing antibodies	Use mAbs if they don't show cross-reactivity against different species or can neutralize whole venoms	• Only limited number of mAbs can be mixed • Identification of perpetrating snake species is not always possible	[65,67,68,70]
Antibody purification	Optimize species specific protocols	Use protocols for venoms produced in other species	Protocols optimized for one species may not be suitable for another	[85]
Endotoxin and albumin removal from serum antibodies	Use chromatography	Use ultrafiltration	Large size of antibodies may block the membrane pores	[87]
Reduce side effects/toxin specific antivenom	Use toxins as immunogens	Select epitopes that are similar to human and animal proteins	To minimize cross-reactivity against host proteins and to identify sequences with minimum immune tolerance	[43,46]
Recombinant expression of venom toxins	Enzymes may be co-expressed for proper folding in prokaryotic systems, or mammalian systems may be used	Express post translationally modified toxins in *E. coli*	Glycosylation, correct disulfide bonding and protein folding are difficult to achieve in *E. coli*	[35,36]
Improve health, lifespan, and immune response of production hosts	• Use purified toxins for immunization, detoxify venom/toxins • Use liquid venoms	Irradiate venom excessively	• Minimum number of antigens would be injected, immunogen toxicity would be reduced • Irradiation may alter toxin structure • Lyophilized and frozen venoms may retain their activity even at high doses of radiation	[26–28]
Antivenom production	Use horses	Use horses continuously	Continuous use for antivenom production may result in inflammation	[130]
Use *in silico* methods for antivenom designing	Careful result interpretation	Depend entirely on *in silico* results	There may be bias in the representation of few toxins of some species over others	[101]
Use ELISA to test antivenom potency	Interpret results carefully	Always correlate absorbance with potency	Toxicity of some venoms may be determined by low immunogenicity toxins	[78]
Administer analgesic to mice before *in vivo* assays	Use morphine, tramadol, or other tested drugs at recommended dose	Administer any analgesic at any dose	The drug may interfere with the test results	[93]

Table 2
Key procedures for regulating the quality of the snake antivenoms [5,90,111].

Category	Production step	Purpose	Requirement(s)/Method(s)
Venom production	Species selection	Represent therapeutically relevant snakes in the targeted region	Epidemiological and clinical information
	Snake selection	Account for intraspecific variability	Physically and geographically diverse snakes
	Snake management	Medical care, deworming, *etc.*	Quarantine room for new snakes
		Maintain animal health	Appropriate diet and feeding schedule
	Venom extraction	Obtain sufficient venom for immunization	Adequate number of snakes depending upon venom yield
	Venom collection	Prevent loss of toxins due to material binding	Pre-validated vessels such as polypropylene or BSA coated
		Prevent degradation	Immediate freezing of extracted venom
	Venom storage	Ensure venom activity	Periodic check against standards for residual moisture, potency
	Reference pool	Account for intraspecific variability	Biochemical and toxicological analysis
	Venom quality	• Ensure ability to elicit immune response • Evaluate antivenom potency	• Biological assays • Characterization of multiple batches
Production host	Animal selection	Ensure animal suitability for antivenom production	• Quarantine, usually for 6–12 weeks before immunization • Physical exam, blood test, vaccination, medical care
	Adjuvant	Minimize adverse effects	Freund's adjuvant used for first injection only
	Injections	Minimize risk of infection	Aseptic conditions
	Throughout immunization schedule	Determine time when animal generates acceptable antivenom titer	Lethality assay, enzyme immunoassays
		Consistent and adequate nutrients	Quality control of food and water
Antibody purification	Animal selection	Check antibody response	Lethality assay, ELISA, toxin specific assay
	Bleeding	Exclude animals in poor health	Medical and blood tests
		Prevent microbial contamination of blood	Animal skin and operator hand must be disinfected
	Before fractionation	Ensure antivenom potency	Inspect for precipitates, hemolysis, contamination, protein content, potency
	Plasma separation	Prevent contamination and minimize endotoxins	Sterile conditions
		Minimize risk of virus transmission	Viral inactivation
	Plasma storage	Preserve plasma quality	Additives in plasma, storage at 2–8 °C
Antivenom		Safety and quality	Physical, biochemical, and toxicological assays

must also be explored to improve the host immune response and production capacities [119]. IgAs should also be examined for use in antivenom therapy, as they are the projected next generation substitute of IgG based biotherapeutics [120,121]. Investigating *in vitro* tests that can simultaneously screen antivenom efficacy against multiple toxins would save time and labour, and thereby allow us to assess each venom-antivenom pair for cross-reactivity research. The generation of transgenic animals expressing one or more venom or toxin specific antibodies in milk can also be a ground-breaking development [122]. Milk would be a non-invasive and economical source of antibodies, and if made suitable for direct patient administration, the need of antibody purification will be eliminated. These animals can be sent to developing nations for in-house antivenom generation without the need for any technical competence, overcoming the restrictions of antivenom shipping and storage while reducing production costs.

Although conventional serum therapy continues to be the only approved treatment for managing SBE, considerable attention of the research fraternity has also been diverted to development of non-antibody based therapeutics. These are poised as the next generation of antivenoms and include plant-based compounds, small molecule inhibitors, synthetic peptides, aptamers, and nanoparticles [6,123–126]. The potential of these alternative therapies can be understood with the orphan drug status being granted to Varespladib-a secretory PLA2 inhibitor by the FDA for SBE treatment, and its approval for phase 2 human clinical trials [127,128]. In future, these products could be combined with conventional or recombinant antivenoms, or used individually, for improved treatment outcomes at reduced costs and better availability.

The year 2019 witnessed the emergence of Covid 19, a pandemic that claimed the lives of millions of people throughout the world. The pandemic seized funds, research focus, personnel, and negatively affected the antivenom market and people's faith in antivenom therapy [129]. However, it also taught us the effectiveness of mass public campaigning to generate awareness and the power of a team-based approach in dealing with complex challenges. Combined efforts at same or higher levels than pre-Covid times, and integration of Covid lessons into SBE management would go a long way in producing ideal BASE antivenoms, thereby significantly reducing the global SBE burden.

CRediT authorship contribution statement

Anurag S. Rathore: Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Ramesh Kumar:** Methodology, Visualization, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing. **Om Shanker Tiwari:** Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anurag Singh Rathore reports financial support was provided by Department of Biotechnology, Ministry of Science and Technology, India.

Data availability

No data was used for the research described in the article.

Acknowledgements

This work was funded by the Center of Excellence for Biopharmaceutical Technology grant (grant number BT/COE/34/SP15097/2015) from Department of Biotechnology, Ministry of Science and Technology, India. RK would also like to acknowledge the Indian Institute of Technology Delhi, India for providing Ph. D scholarship.

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