Review

Antivenoms for the treatment of snakebite envenomings: The road ahead

José María Gutiérrez [a,*], Guillermo León [a], Thierry Burnouf [b]

[a] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica
[b] Human Protein Process Sciences, Research and Development, 18 rue Saint Jacques, Lille, France

ARTICLE INFO

ABSTRACT

The parenteral administration of antivenoms is the cornerstone of snakebite envenoming therapy. Efforts are made to ensure that antivenoms of adequate efficacy and safety are available world-wide. We address the main issues to be considered for the development and manufacture of improved antivenoms. Those include: (a) A knowledge-based composition design of venom mixtures used for immunization, based on biochemical, immunological, toxicological, taxonomic, clinical and epidemiological data; (b) a careful selection and adequate management of animals used for immunization; (c) well-designed immunization protocols; (d) sound innovations in plasma fractionation protocols to improve recovery, tolerability and stability of antivenoms; (e) the use of recombinant toxins as immunogens to generate antivenoms and the synthesis of engineered antibodies to substitute for animal-derived antivenoms; (f) scientific studies of the contribution of existing manufacturing steps to the inactivation or removal of viruses and other zoonotic pathogens; (g) the introduction of novel quality control tests; (h) the development of *in vitro* assays in substitution of *in vivo* tests to assess antivenom potency; and (i) scientifically-sound pre-clinical and clinical assessments of antivenoms. These tasks demand cooperative efforts at all main stages of antivenom development and production, and need concerted international partnerships between key stakeholders.

1. Introduction

Envenomings due to snakebites are a major neglected tropical disease affecting predominantly poor people living in rural settings in Africa, Asia, Latin America and Oceania [1–3]. Although accurate statistics are often unavailable, the yearly world-wide occurrence of envenomings may range between 421,000 and 1,841,000, provoking from 20,000 to 94,000 fatalities [4]. In addition, a significant proportion of these accidents end up with permanent tissue damage and sequelae, with high socioeconomic and psychological impacts [1,3]. The parenteral administration of antivenoms of animal origin is the cornerstone therapy of snakebite envenomings since the first *serum antivenimeux* was developed in the last decade of the 19th century [5].

Antivenoms are currently manufactured by at least 45 laboratories in all continents (see http://apps.who.int/bloodproducts/snakeantivenoms/database/). The basic antivenoms production process involves the immunization of animals, usually horses but sometimes other species, with venoms from a single or various snake species. After collection of blood or plasma, the plasma is fractionated to extract and purify the active immunoglobulin substances. Depending on the fractionation protocol, three types of active antivenoms substance are obtained: (a) whole IgG, currently isolated using either ammonium sulphate or caprylic acid [6,7]; (b) F(ab')$_2$ fragments, prepared by pepsin digestion and ammonium sulphate or caprylic acid fractionation [8,9]; and (c) monovalent Fab, prepared by papain digestion and ammonium sulphate fractionation [10]. After filling in the final containers, antivenoms may be kept liquid or be freeze-dried to increase their stability [11]. Some manufacturers have introduced process variations, such as chromatography and pasteurization, to improve purity and/or viral safety [9]. Large heterogeneity in technologies used, scale of production, staff qualification, quality control protocols, and extent of implementation of good manufacturing practices (GMP) are found among manufacturers. A major step forward as a guideline for improving antivenoms quality world-wide is the recent WHO publication entitled "Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins" [11], which offers manufacturers and regulators with practical recommendations on selection and preparation of venoms, maintenance of snakes, immunization protocols of horses, collection and storage of blood/plasma, plasma fractionation methods, quality control tests, viral safety measures, pre-clinical and clinical assessment of efficacy and safety, and general regulatory approaches. These

* Corresponding author. Tel.: +506 2229 3135; fax: +506 2292 0485.
E-mail address: jose.gutierrez@ucr.ac.cr (J.M. Gutiérrez).

guidelines set the stage towards standardization of antivenom manufacture and quality control.

Although most current antivenoms exhibit improved efficacy and safety profiles compared to former generation products, issues still need to be addressed for further improving antivenom design and manufacture. These issues demand concerted efforts in research and development, and the translation of research findings into the technological realm for preparing higher quality antivenoms. The present work highlights some of the on-going technical development areas as well as issues to be addressed for improving antivenom technology. Additional aspects, such as production economics, purchase and distribution systems, accessibility in developing countries, proper training of health workers in the diagnosis and treatment of snakebite envenomings, and correct use of antivenoms in the clinical setting, although highly important, are beyond the scope of this work and, therefore, not addressed here.

2. Towards a knowledge-based approach in the design of immunization mixtures: taking advantage of research in venom biochemistry, immunochemistry and toxicology

Unlike antigens used for the production of tetanus and diphtheria antitoxins, and rabies immunoglobulins, the venoms of snakes are characterized by an astonishing inter- and intraspecies variability [12,13]. Consequently, the selection of venoms for inclusion in immunizing mixtures is critical in antivenom production [11,14]. Many decades ago, the selection of venoms for immunization was often based on toxic profile of venoms and broad taxonomic considerations, without considering biochemical and immunochemical analyses of venoms. Studies have shown that, whereas for some venoms the designs were appropriate, for others they were misleading, generating antivenoms ineffective against the venoms of some medically-relevant snake species. In the last decades, the knowledge of venom biochemistry, toxicology and immunology has grown exponentially, providing important information for the improvement of antivenoms. This gap between basic knowledge of venom composition and effects and practical antivenom production should be filled to enhance antivenoms quality and efficacy.

Current proteomics tools make detailed characterization of venoms now possible (see [15–17] for reviews). In addition, toxicological and enzymatic assays can be used to characterize venom activities. Moreover, routine electrophoretic and chromatographic analytical procedures allow the investigation of individual, ontogenetic and regional variability in venom composition. This set of analytical tools is currently used by many laboratories to characterize venom composition and variability. One application is the preparation of national or regional reference venoms for species with wide distribution and significant medical impact. Examples of such wide distributed species are *Bitis arietans* in Africa [18], *Daboia russellii* in Asia [19], *Bothrops atrox* [20] and *B. alternatus* [21] in South America, *Crotalus viridis* in North America [22], and *Vipera aspis* in Europe [23]. In these cases, a conspicuous pattern of regional variation in venom composition requires careful analysis to select the most adequate mixture of venoms of various geographical origins to prepare representative reference venom pools. Such pools are likely to elicit an immune response effective in the neutralization of venoms from different regions and are also useful for quality control of antivenoms.

Venoms of Central and South American *Crotalus* species illustrate the usefulness of these analyses. The venom of adult specimens of *C. simus*, distributed in Central America, is abundant in metalloproteinases and poor in the neurotoxic dimeric phospholipase A_2 complex 'crotoxin' [24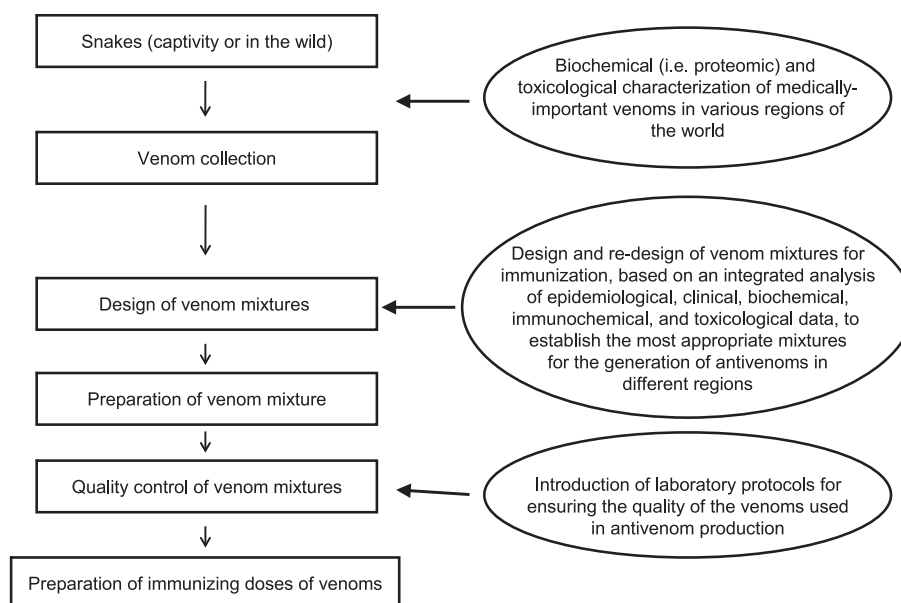]. In contrast, the venom of neonate specimens of *C. simus*, and those of neonate and adult specimens of the South American subspecies of *C. durissus* present low amounts of metalloproteinases and high concentration of crotoxin, therefore having higher toxicity and provoking a different pathophysiology compared to *C. simus* venom [13,25–27]. Moreover, some *C. durissus* populations contain the toxin crotamine, which provokes contracture and spastic paralysis, whereas other populations lack this protein [13,28,29]. Such proteomic information allows a knowledge-based design of the composition of immunizing mixtures for the neutralization of *Crotalus* sp venoms in Central and South America. In this case, the mixture should include venom(s) containing high amounts of crotoxin and crotamine, e.g. from some populations of South American *C. d. terrificus*, and venom(s) containing high amounts of metalloproteinases, e.g. from adult *C. simus*. Both types of venoms contain thrombin-like serine proteinases, which are necessary to elicit the formation of antibodies able to neutralize coagulopathy. Biochemical analyses have therefore paved the way for scientifically-based design of the most adequate mixture of venoms for immunization, making possible the generation of antivenoms effective for the treatment of rattlesnake envenomings in Central and South America.

When venom mixtures are designed on the basis of regional variation analysis, such mixtures should be tested to demonstrate that they evoke an antibody response able to neutralize key toxic activities in medically-relevant venoms. Such testing should be performed by combining neutralization assays, e.g. neutralization of lethality and other activities, and by immunochemical analysis of reactivity [16]. In recent years, proteomic analytical tools have been adapted to assess the immune reactivity of antivenoms against individual venom components [16,29]. This methodological approach, named 'antivenomics', permits the classification of toxins in a venom as C-toxins (completely immunodepleted by an antivenom), P-toxins (partially immunodepleted by an antivenom), and N-toxins (not depleted by an antivenom) [17]. Antivenomics, in combination with the study of neutralization of toxic and enzymatic activities of venoms by antivenoms [11,30], are highly useful to test whether a mixture of venoms elicits an antibody response capable of recognizing and neutralizing the most relevant components in a venom (see, for example, [31–33]); it is necessary to analyze whether the traditional immunizing mixtures used in the production of many antivenoms are the most adequate or whether they need to be revised based on biochemical and toxicological data. Likewise, the design of new antivenoms should take advantage of this arsenal of analytical resources.

Bioinformatic tools should be also incorporated in the analysis of venom variability and in the selection of the most appropriate venom mixtures for immunization. The proteomic and transcriptomic information gathered on snake venoms allows for the identification of common antigens and potential toxin epitopes [34,35]. This information, in turn, can be used to predict which combinations of venoms elicit an effective immune response against the most relevant toxins in a venom or in a group of venoms. Evidently, applying proteomic, transcriptomic and bioinformatic methods in the design of venom mixtures for antivenom production demands the collaboration between research and antivenom development/manufacture groups. Such cooperative scenario should be promoted by funding agencies, health authorities and manufacturers alike (Fig. 1).

3. The selection of the most effective venom mixtures for immunization: integrating epidemiological, clinical and immunological information

The design of optimal venom mixtures for immunization should also be based on epidemiological and clinical criteria. Renewed research efforts of snakebite epidemiology and clinical manifestations

Snakes (captivity or in the wild)

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Venom collection ← Biochemical (i.e. proteomic) and toxicological characterization of medically-important venoms in various regions of the world

↓

Design of venom mixtures ← Design and re-design of venom mixtures for immunization, based on an integrated analysis of epidemiological, clinical, biochemical, immunochemical, and toxicological data, to establish the most appropriate mixtures for the generation of antivenoms in different regions

↓

Preparation of venom mixture

↓

Quality control of venom mixtures ← Introduction of laboratory protocols for ensuring the quality of the venoms used in antivenom production

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Preparation of immunizing doses of venoms

Fig. 1. Suggested development tasks (circles at right) in venom collection and preparation, design of venom mixtures for immunization, and quality control of venoms (boxes at left) to improve quality, safety and efficacy of antivenoms.

of envenoming are required. This is essential to establish whether a venom needs to be included in a mixture for immunization, and whether monospecific or polyspecific antivenoms are required [11]. For instance, for species that inflict accidents with clinical manifestations that allow the identification of the offending snake, a monospecific antivenom may be indicated. Such is the case of the crotalic antivenoms produced in various South American laboratories against the venom of *C. durissus* (see http://apps.who.int/bloodproducts/snakeantivenoms/database/). In contrast, species of *Bothrops* in Latin America induce a similar picture of pathological and pathophysiological manifestations, thus requiring polyspecific antivenoms [27,32]. Similarly, a new polyspecific antivenom for sub-Saharan Africa is prepared by immunizing horses with a mixture of the venoms of two viperid species, *Echis ocellatus* and *Bitis arietans*, and one elapid species, *Naja nigricollis* [36]. The rationale behind this design is that, unlike other cobra species whose envenomings are characterized by neurotoxic manifestations, those by *N. nigricollis* and related spitting cobras are primarily associated with local tissue damage. The difficulty in discerning between viperid and elapid bites in this case justified the preparation of an antivenom that would neutralize both types of venoms.

Novel epidemiological and clinical findings may reveal the occurrence of envenomings by species hitherto unknown to represent a relevant health hazard, or presenting unusual clinical manifestations. Examples are *Hypnale hypnale* [37], *Bungarus candidus* [38] and *Bothrops lanceolatus* [39]. Such information may justify including these venoms in immunizing mixtures used for polyspecific antivenom preparations or, if considered necessary, for the development of new monospecific antivenoms. Likewise, when preparing pools of immunizing venoms, evidence of geographical variation in the clinical presentation of envenomings, such as by *Daboia russellii* in various regions of Asia [19,40] should be considered, in conjunction with the biochemical and immunological criteria discussed above. Therefore, a careful and knowledge-based analysis, integrating biochemical, immunological, epidemiological and clinical information, should be performed for designing immunizing mixtures used in antivenom manufacture in different regions of the world. A selected immunizing venom mixture should

be validated by preparing a pilot antivenom batch and by testing it by neutralization assays against the targeted venoms.

4. Scientific names matter: the importance of keeping up with taxonomic changes

Snake taxonomy and systematics are active research fields. Changes in species and genera names are regularly proposed following ever-growing morphological, molecular and bioinformatics data (see, for example [41]). When such taxonomic modifications occur in medically-relevant species, they carry relevant implications for antivenom manufacture as well as for toxinological research and envenoming treatment protocols [11]. This affects the identification of snakes in collections and of venom samples used in research and in antivenom manufacture and quality control. This issue is particularly relevant when antivenom production laboratories rely on external sources for venom supply, thus depending on the capacity of such sources to correctly identify the species, and the geographical origin of specimens from which venoms are collected. The potential problem of species misidentification, due to using old scientific names, can be circumvented by close interaction between groups working in snake taxonomy and systematics and manufacturing laboratories, and by an appropriate traceability of venom pools used in production. Regular publication of reviews, by joined teams of taxonomists and clinicians, summarizing the most relevant taxonomic changes involving medically-relevant snake species, facilitates such flow of information [11,42]. Likewise, the involvement of herpetologists in venom and antivenom-producing facilities ensures correct scientific names in species identification and in antivenom labels.

5. Improving the selection, management and immunization protocols in animals used for antivenom production

5.1. Selection and clinical assessment

Antivenoms are produced by immunizing animals with whole venoms or isolated venom components, and this approach is likely

to continue. However, in many instances animal selection, immunization, bleeding and clinical control are performed following a rather empirical and poorly-controlled process. The WHO antivenoms guidelines include a section dealing with animal selection and care, emphasizing this critical aspect of antivenom production [11]. Future efforts should focus in areas such as:

(a) The development and widespread distribution of immunodiagnostic and nucleic acid testing kits to detect relevant microbial infections in animals used in immunization. The collaboration between veterinary immunologists and antivenom producers should be promoted to facilitate the development, validation and implementation of diagnostic kits adapted to the economics and specifics of antivenom manufacture. This would contribute to the proper selection of animals introduced in immunization programs.

(b) The establishment of validated protocols for the follow-up of the health status of immunized animals, including veterinary care, use of clinical laboratory methods and implementation of computerized tools for traceability of health controls. Studies on the clinical and laboratory alterations occurring in animals immunized with different venom mixtures should be promoted to strengthen knowledge-based maintenance programs.

5.2. Improving animal immunization for antivenom production

Animal immunization is a critical stage in antivenom production. Essentially all quality factors, i.e. efficacy, safety, availability and cost, largely depend on the neutralizing activity reached by the plasma of immunized animals. High neutralizing titers result in benefits such as: (a) higher volume of antivenom, (b) higher potency, (c) lower total protein formulation, and, consequently, (d) more affordable products [43]. Therefore, procedures to obtain hyperimmune plasma need to be optimized. This should include considerations to (a) animal species used, (b) preparation and control of venoms used as immunogens, (c) immunization schedules, and (d) validation of antibody response assays (Fig. 2).

5.2.1. Animal species used as source of immunoglobulins

Horses, and in some cases sheep and donkeys, are currently used to raise hyperimmune plasma for antivenom production [44–46]. Most experience is with horses, and they will likely remain predominantly used in the future. However, other species that could provide satisfactory high-yield antibody responses may be highly convenient in some regions. For instance, camelids (such as camels or llamas) can adapt better than horses in parts of Africa, Middle East and highlands of South America [47,48]. Immunization of hens has also been proposed as a low cost, high-yield procedure for preparing avian antibodies [49–52]. Investigating the characteristics of the immune response of these species to snake venoms is necessary [53], as well as considering the possible risks of allergy to egg or serum proteins. Even with horses, the basic immune response to venoms has received little attention and demands future studies aiming at understanding antigen presentation, kinetics of response, immune regulation by venom components, development of antibody affinity, genetic aspects of the immune response, etc. Clearly, cooperation between antivenom producers, veterinarians and immunologists should be fostered to clarify these issues.

Immunoglobulins from different species have different physicochemical characteristics to be considered for antivenom production. Horses respond to venom immunization by generating predominantly ∼180 kDa [54] IgG(T) antibodies [55]. Camelids produce two types of antivenom antibodies: (a) heterotetrameric IgGs (∼160 kDa), corresponding to the IgG1 subclass, composed by two identical heavy chains and two identical light chains, and (b) homodimeric IgG (∼100 kDa), the heavy chain antibodies, corresponding to the IgG2 and IgG3 subclasses, made of two identical heavy chains without light chains [56]. The stability of these camelid IgGs differ [53]. In turn, hens produce ∼180 kDa IgY antibodies composed by two heavy chains and two light chains [50]. These various immunoglobulins should be compared for neutralizing capacity against venom toxins, stability at different temperatures, storage conditions, and tendency to induce adverse reactions [53,57,58]. For instance, high degree of glycosylation of horse IgG(T) was thought to induce adverse reactions [59,60], but this is not supported by a recent clinical trial comparing equine and ovine-derived whole IgG antivenoms [61].
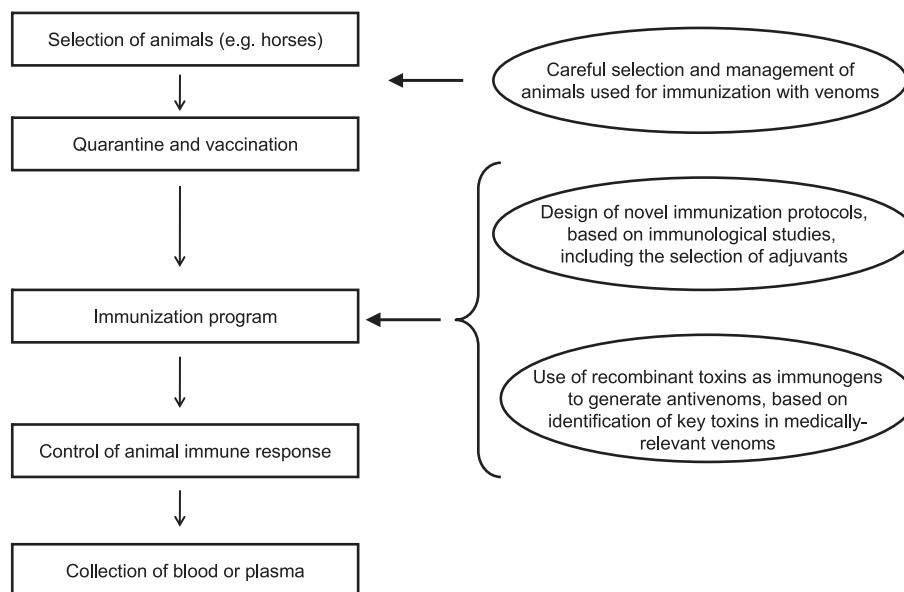
Fig. 2. Proposed development tasks (circles at right) in. animal selection and care, immunization and bleeding programs (boxes at left) to improve quality, safety and efficacy of antivenoms.

Research and development is required to improve the bleeding processes used by production laboratories. Automated plasmapheresis should be considered in place of the traditional whole blood collection protocols ("manual apheresis") since it provides a more controlled and aseptic alternative to obtain equine plasma [62]. Likewise, the clinical effects of bleeding programs on immunized animals should be investigated to develop protocols which maximize plasma yield without harming animals.

5.2.2. Manipulating the antigen carefully: improvement of venom preparation and use

Proper collection, handling, storage and preparation of venoms for immunization are critical to ensure an adequate immune response. This is particularly relevant considering that venoms are often prepared by independent laboratories. Collection and storage procedures of venoms should be validated to ensure absence of physicochemical alterations of venom proteins that could potentially affect the efficiency of the immunization [11,63]. Physicochemical characteristics of venom toxins can be affected by inherent venom instability, e.g. due to proteases [64], and/or stress produced during venom drying. Loss of important epitopes may affect venom antigenicity, generating a poor immune response. Therefore, processes used to obtain and stabilize venoms (i.e. desiccation or freeze-drying) should be properly validated, and the pharmacological and biochemical properties of venom batches used as immunogens should be characterized. In this context, protocols aimed at assessing venom quality are critical in antivenom-producing laboratories (see [11] for details).

Some studies have described different methods to detoxify venoms for immunization [65–68], a process that may induce loss of antigenicity. Some producers use detoxified venom for immunization, especially when dealing with highly toxic venoms. This is necessary in high-dose immunization schemes that lead to significant damage to animals when using native venoms. However, since snake venoms are highly complex mixtures of many antigens, the effect of such detoxifying steps on different toxins should be carefully evaluated, a task that requires detailed biochemical knowledge and analysis of the venoms. As discussed below, the trend is to reduce the total amount of venom injected in animals, taking advantage of novel immunization strategies. This approach should be promoted to achieve good immune responses with native venoms, thus avoiding the need for venom detoxification.

Snake venom antigenicity may also be altered by other venoms used as co-immunogens for producing polyspecific antivenoms. Immunosuppressive activity has been demonstrated when using *Lachesis muta* [69] and *Crotalus durissus terrificus* [70,71] venoms. This should be considered and investigated by laboratories producing polyspecific antivenoms. A case by case analysis, where different combinations of venoms are tested for occurrence of negative immunomodulation, is recommended. Decisions could then be drawn on whether a polyspecific antivenom should be prepared by immunizing animals with a venoms mixture or by mixing plasmas of animals immunized with the corresponding single venoms [11]. Likewise, the immunological mechanisms behind such immunomodulatory events should be investigated to take measures regulating these negative interactions.

5.2.3. Adjuvants and immunization schemes

Adjuvants are used to improve the antibody response towards immunogens [72]. The most frequently used are aluminum salts and Freund's adjuvants [11,36,73], Freund's complete and incomplete adjuvants being the most effective. However, since they induce tissue damage at the site of injection, their use is limited to the animal priming and first immunogen boosters [43]. It is necessary to explore new, less toxic adjuvants. Examples include liposomes [74] and stimulation of immune mechanisms associated with cell activation and cytokine production [75]. Cross-talk between vaccine developers and antivenom producers should be encouraged to harness knowledge generated in the vaccine field in the development of new adjuvants for application in antivenom manufacture.

Likewise, a critical assessment of current immunization schemes should be performed. This is a key area in antivenom manufacture that has received relatively little attention; in many cases, such protocols were developed decades ago and have remained unchanged. A number of old immunization schemes were based on the administration of rather large doses of venom [76,77], with the consequent effect on animal health and, possibly, on the generation of immune tolerance associated with a poor antibody response. Antivenom producers and research and development groups should design novel immunization schemes, analyzing variables such as venom doses, number of immunizations, time interval between venom injections, number of anatomical sites injected and volume of injection per site, among others, as well as exploring novel adjuvants at different stages of the immunization schedule. Recent advances include the use of low doses of venom [78] and low dose, low volume, multi-site protocols [79], with highly promising results. The future immunization protocols for antivenom production will likely be based on low doses of native venoms, administered with novel adjuvants, and injected at anatomical sites selected to maximize the contact between venom antigens and the immune tissues and cells. Here again, cooperative research is urgently needed.

5.2.4. The assessment of antivenom antibody response

Development of new immunization protocols and routine assessment of antivenom antibody response in immunized animals demand laboratory determination of (a) the immune response to facilitate the selection of the best responders, and (b) the optimal time of bleeding. In many laboratories, such assessment is performed by routine potency test using mice. This requires many mice, especially if the immunological response is tested at individual level. If, on the other hand, pools of plasma are tested, the inter-individual differences in the antibody response are masked, with the possibility of keeping poor responders within the group. Hence, it is desirable to develop *in vitro* tests to follow up the individual immune response of animals to venoms. Such tests should correlate with the traditional potency assay in mice to ensure that the selection of good responders and bleeding times are based on effective neutralizing antibody responses (see Section 9.2).

6. Plasma fractionation: towards antivenoms of high physicochemical quality, satisfactory yield and low cost

Antivenoms are manufactured by fractionating the plasma of immunized animals to generate products containing either whole IgG or immunoglobulin fragments F(ab')$_2$ or Fab [11,44,46]. Antivenoms from different laboratories vary in physicochemical characteristics, such as total protein concentration, level of protein aggregates and non-IgG plasma proteins, and presence of Fc fragment in whole IgGs. These may influence the risk of early and late adverse reactions, the frequency of which greatly differ among products, from 6% [80] to higher than 80% [81–83]. Late adverse reactions likely correspond to type III hypersensitivity, mediated by the formation of immune complexes between antivenom proteins and human antibodies generated against antivenom. The incidence of these reactions seems related to the total amount of foreign protein administered [84]. Poor tolerability of some whole IgG antivenoms was long been thought to be due to the presence of Fc. However, IgG and F(ab')$_2$ antivenoms of good physicochemical profile were found to induce a similar low incidence of early

adverse reactions [61,82,85], whereas antivenoms with poor physicochemical features, i.e. turbidity, high content of protein aggregates or non-IgG contaminating proteins, exhibit low tolerability [81–83,86–88]. The mechanisms responsible for early adverse reactions, although likely dependent on physicochemical characteristics [44,89], have not been completely elucidated [44,46], a subject that requires renewed research efforts.

6.1. State of the art: basic technological platforms for the purification of antivenom active substances

6.1.1. Purification of whole IgG

Salting-out with ammonium or sodium sulfate has long been the main whole IgG purification procedure. A typical protocol involves two precipitation steps at two different salt concentrations, in addition to 'euglobulins' removal by diluted acid precipitation [7]. However, when not properly standardized, this fractionation protocol yields a product with high content of non-Ig proteins, such as albumin and non-Ig globulins, as well as excessive protein aggregates [6], with a detrimental impact on safety [88]. Whole IgG antivenoms of good physicochemical profile and purity are readily obtained by caprylic acid (octanoic acid) precipitation of non-IgG plasma proteins [6,90]. Caprylic acid fractionation involves slow addition of caprylic acid to undiluted plasma. Precipitated protein is separated from the solution by filtration or centrifugation. Immunoglobulins are then diafiltered, formulated, sterilized by filtration, and dispensed in final containers [6,11]. Caprylic acid fractionation generates antivenoms of relatively high purity with little aggregate content, in part because the IgG is not precipitated during processing. The process yield is ~60% [6]. The efficacy and safety profiles of such product have been demonstrated in clinical trials [61,82,88,91].

6.1.2. Purification of F(ab')$_2$ fragments

Most manufacturers use optimized versions of the F(ab')$_2$ antivenom production protocol published by Pope in 1939 [8,92]. This technique involves the digestion of plasma proteins by pepsin, usually at 1 g/L and pH 3.3 for 1 h at 30–37 °C [11], although different protocols yield similar results. After pH adjustment with NaOH, a solution of ammonium sulphate is added under stirring to reach 12% (w:v) final concentration. The precipitate is eliminated by either filtration or centrifugation, and the solution is submitted to 56 °C heating for 1 h ('thermocoagulation'). The mixture is filtered or centrifuged to eliminate the precipitate. The pH is adjusted to neutral value with NaOH, and a solution of ammonium sulphate is added under stirring to reach 23% (w:v) concentration, to precipitate F(ab)$_2$ fragments [11]. After an additional filtration, or following centrifugation, the F(ab')$_2$ precipitate is dissolved, diafiltered, formulated, sterile-filtered, and dispensed [11,46]. Caprylic acid precipitation of non-F(ab')$_2$ proteins can be introduced in substitution of ammonium sulphate precipitation, resulting in improved yield [93,94].

6.1.3. Purification of Fab fragments

Monovalent Fab fragments antivenoms have been developed by some manufacturers [95–97]. They are usually obtained from sheep hyperimmune plasma. After separation of plasma from blood cells, immunoglobulins are precipitated by ammonium or sodium sulphate. The precipitate, containing the IgG fraction, is then dissolved in buffered sodium chloride solution at neutral pH. Papain is added to the Ig solution and a 37 °C-18–20 h digestion is performed [11]. Reaction is stopped by iodoacetamide. The product is diafiltered and equilibrated with a buffered isotonic sodium chloride solution. Afterwards, the preparation is submitted to anion-exchange chromatography, formulated, sterilized and dispensed in the final containers [10,11,46].

6.2. Additional processing steps to improve the purity of antivenoms

Additional purification steps to increase the purity of antivenoms can be implemented, some of which are already used [10]. For instance, DEAE-Sepharose or Q-Sepharose Fast Flow anion-exchange gels or quaternary ammonium cellulose microporous membranes can be used [90,92,94]. Usually, the conditions are designed to adsorb contaminants, whereas the active principles pass through the column or the membrane [10,92]. Anion-exchange chromatography has also been suggested for the removal of bacterial lipopolysaccharides [98]. The use of affinity chromatography has been described [99,100]. When venoms are immobilized on chromatographic gels and antivenoms are passed through, only antibodies against venom components are retained, greatly increasing the purity of these preparations and reducing the load of IgGs directed to non-venom antigens. The use of affinity columns demands a meticulous validation of the cleaning procedures, as well as control of absence of venom ligand leakage; likewise, the conditions for eluting the antibodies may affect their stability. Other alternatives such as hydrophobic interaction-based membrane adsorption have been advocated [101]. Research should focus on the development and adaptation of novel purification steps, taking advantage of developments in the field of protein chromatography. Nevertheless, these studies should include a cost-benefit analysis to ensure that new production steps do not significantly affect the yield nor result in excessive increments in the production costs and price of antivenoms. Economic analyses in the development and design of antivenoms is needed (see for example [102]).

6.3. Reducing the problem of protein aggregation in antivenoms

Immunoglobulin aggregates have been associated with possible adverse reactions because of their ability to simulate immune complexes and activate the complement system [46]. Protein aggregation, leading to turbidity, is common in antivenoms products with high incidence of adverse reactions [88]. Therefore, manufacturers should assess fractionation protocols to detect steps that promote protein instability leading to aggregation. Those may include: (a) precipitation of IgGs or IgG fragments, followed by an incomplete solubilization of the precipitate. This might occur when proteins are precipitated by ammonium salts in salting out procedures; (b) instability induced by heating, especially during 'thermocoagulation'; (c) possible formation of idiotype-anti-idiotype complexes, when using plasma pools from a large number of individuals, a phenomenon described in human IgG preparations [103], but not investigated for antivenoms; (d) effect of excipients on antivenom stability (phenol, for instance, exerts a denaturing effect on antivenom IgGs [104,105]); and (e) deficient freeze-drying protocols, resulting in protein stress leading to aggregation and turbidity. The causes of protein aggregation and turbidity should be carefully examined by antivenom producers to optimize fractionation protocols. Likewise, quality control of antivenoms should include turbidity testing (see Section 9.1).

6.4. Improving antivenom stability

Snakebite envenoming predominantly occurs in tropical countries with high temperature and humidity (climatic zone IV). Antivenoms should be formulated to maintain product characteristics and guarantee safety and efficacy along shelf-life, including distribution and storage. The fact that many antivenoms used in tropical countries are formulated as liquid products, together with a deficient cold chain system in many areas, represents a problem that demands consolidated research and development efforts to generate liquid antivenoms of higher stability. Among several

strategies, freeze-drying has been considered the preferred option to ensure antivenom stability. Freeze-drying is a relatively complex procedure involving freezing, sublimation and desorption [106]. Although robust methods have been developed and applied for antivenoms, the thermal stress to which proteins are submitted during freeze-drying can promote denaturation if the process is not properly monitored. Efforts should be directed at evaluating and improving protocols used in the preparation of freeze-dried antivenoms, adapting them to each particular product [107]. Likewise, the need for freeze-dried versus liquid antivenoms calls for careful case by case analysis since the former have a higher production cost. In addition, research and development efforts should target novel liquid antivenom formulations, using various excipients and pHs, with enhanced protein stability, thus possibly allowing the storage of liquid antivenoms at temperatures encountered in tropical regions. This is a research area of great potential, and promising advances have been made [108,109].

6.5. The issue of preservatives

As indicated in the WHO Guidelines [11], preservatives to prevent bacterial and fungal contamination should be kept to a minimum during plasma storage and fractionation, and should never substitute any GMP aspect. Phenol and cresols are added to the final product by many manufacturers [11], especially for liquid formulations [11]. The potential effect of preservatives on the active substance, or its interactions with excipients, should be carefully analyzed by manufacturers. To minimize protein aggregation induced by preservatives, producers should perform tests of efficacy and determine the minimal preservative concentration having bacteriostatic activity. In addition, new preservative substances generating minimum stress to antivenom IgGs should be considered. Optimally, however, manufacturers should implement operating procedures and fractionation protocols able to prevent microbial contamination, so as to avoid the need of preservative, as is now the standard in the production of liquid intramuscular or intravenous human plasma-derived immunoglobulin [110]. Decisions to stop the addition of preservatives to

antivenoms products should however carefully consider the possible virucidal benefits of these compounds, at least on enveloped viruses, as discussed below. Fig. 3 summarizes the most important tasks that need to be undertaken for improving the plasma fractionation protocols and quality control of antivenoms.

6.6. Back to the basics: the need to implement GMP in the manufacture of antivenoms

For various reasons, some antivenom manufacturers have been lagging in implementing the basic principles of Good Manufacturing Practices (GMP). Manufacturers should implement GMP to their operations, including bleeding protocols, water provision system, design of laboratories and equipment, sanitation procedures, manufacturing process, and documentation and training of the staff involved in all steps of the manufacturing process [11]. A proper quality assurance of antivenom production should encompass routine quality control analysis of the in-process products, raw materials and equipments during fractionation, and all operations should be traceable. Such analyses are aimed at assessing the concentration of the active principle and excipients along the process, as well as preventing possible contamination by microorganisms. In-process controls should prevent failures during manufacture and the results should be used to determine the causes of failures and prevent reoccurrence. Although GMP principles are outside the scope of this work, international cooperative efforts should be implemented to help antivenom manufacturers to adhere to GMP outlined in WHO and other organizations guidelines.

7. Validating the pathogen safety of plasma-derived antivenoms based on scientific evidence

7.1. Viruses

There is no report of transmission of viruses, or other zoonotic diseases, by any animal plasma-derived antivenoms (or other antisera) regardless of their production method [11,111,112]. Most
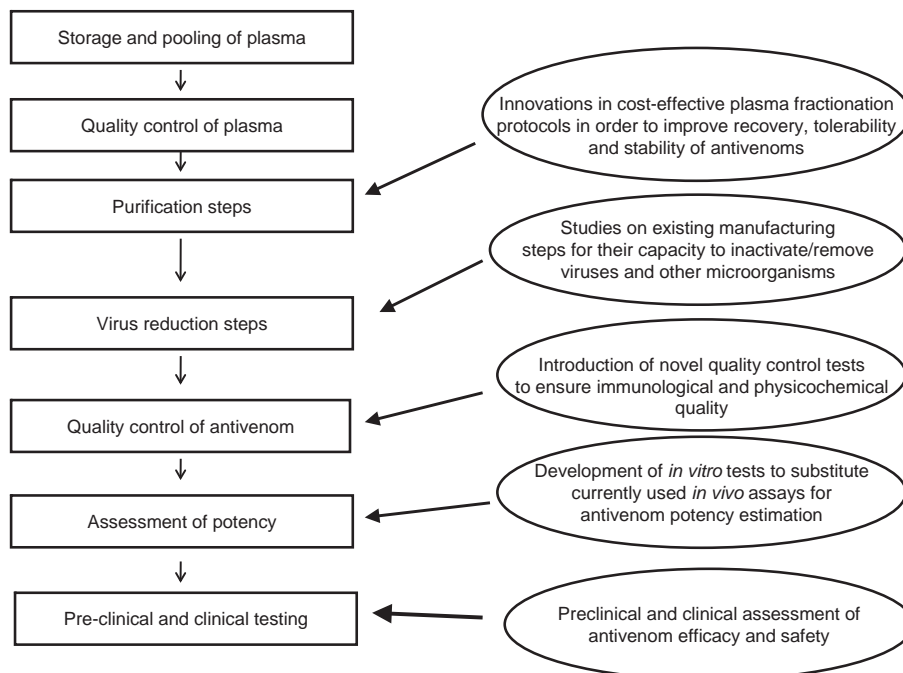

Fig. 3. Proposed development tasks (circles at right) in hyperimmune plasma storage and fractionation, quality control and pre-clinical and clinical assessment (boxes at left) to improve quality, safety and efficacy of antivenoms.

production processes do not however include deliberately added viral reduction steps. As such, whether the apparent safety profile of antivenoms is due to production processes contributing to robust viral inactivation or removal, or is a consequence of improper epidemiological surveillance systems in the countries where these products are mostly used, is unclear. Increasing scientific evidence, however, suggests that several pre-existing purification steps of antivenoms contribute to robust inactivation or removal of viruses. This possibility is reinforced by the fact that similar steps used in the manufacture of human IgG products have gone through formal validation studies demonstrating their robustness for viral reduction [112]. For instance, caprylic acid treatments used in the production of F(ab)'$_2$ fragments were found to inactivate/remove lipid-enveloped viruses [113,114] and some non-enveloped viruses [114]. Pepsin digestion of plasma at low pH (pH 3.2) to generate F(ab)'$_2$ can inactivate lipid-enveloped viruses [112,115] and may also affect some non-enveloped viruses [112]. Depth-filtration steps with filter aids to clarify pepsin-digested IgG can remove lipid-enveloped and non-enveloped viruses [116]. Dedicated heat-treatments in the liquid state at 58–60 °C can inactivate lipid-enveloped viruses and some non-enveloped viruses [114]. Specific viral removal steps on nm-size membranes, known as nanofiltration, can ensure robust removal of viruses [117,118]; this approach could be more readily used for low molecular mass IgG fragments than for whole IgG. Phenol and cresol, at 0.25–0.35%, used as preservatives both in plasma and final products are both lipophilic agents and may have, in principle, the capacity to inactivate lipid-enveloped viruses [11], potentially contributing to the apparent viral safety profile of antivenoms. Recent experimental studies have shown the feasibility of introducing a dedicated solvent-detergent (S/D) treatment of horse plasma prior to caprylic acid fractionation of a whole IgG antivenom used to treat viperid snakebite envenoming [119]. There was no detrimental impact on the yield, quality and potency of the product [119], indicating that this technology could possibly be implemented, if needed, to improve the safety of whole IgG antivenoms against lipid-enveloped viruses. Preliminary experimental evidence also suggests that a pH 4 treatment of a purified whole IgG antivenoms, as applied to human intravenous IgG [120], can inactivate lipid-enveloped viruses (Segura et al. in preparation). Finally, the role potentially played by phenol and cresol in the inactivation of lipid-enveloped viruses needs to be assessed.

A better understanding of the robustness of existing manufacturing steps in contributing to viral safety of antivenoms is needed. This would avoid any potentially unjustified introduction of dedicated viral reduction treatments that could affect the production cost and yield of antivenoms as well as, potentially, their efficacy. Also, by gaining scientific understanding of the viral reduction effects of current manufacturing steps, producers could ensure their appropriate implementation and monitoring at large-scale [112], in compliance with GMP. Viral validations studies following current international guidelines [121] by antivenoms manufacturers should be strongly encouraged and facilitated. As manufacturers in developing countries often lack the infrastructure and know-how to design and perform such validation studies locally, support from international organizations, regulatory authorities and experienced Virology laboratories is needed to validate "generic" production steps of antivenoms and the potential contribution of phenol and cresol to viral safety.

7.2. Prions

The fact that few antivenoms are from the plasma of ruminant species, such as sheep, raises the theoretical risk of transmission of prions. Experimental prion clearance studies by spiking experiments are encouraged [11], as has been done for human plasma fractionation [122] for a better understanding of the capacity of sheep-derived antivenoms production steps to remove prion proteins.

8. Recombinant toxins, monoclonal antibodies and genetically-engineered antibodies as alternatives for antivenom production

The basic methodology for the production of antivenoms, as outlined above, is likely to remain the predominant manufacturing platform for many years to come. However, developments in cellular and molecular biology and genetic engineering may provide novel tools to improve antivenoms or to develop new product generations. Some of the areas applicable to antivenom manufacture in the future are highlighted below:

8.1. Immunization with DNA or with recombinant toxins

The production of antivenoms demands relatively high amounts of venoms for immunization and quality control. For abundant snake species, the acquisition of venom is not a problem. However, some species are difficult to collect and to maintain in captivity, either because of national conservation policies or because of drastic reductions in their natural populations. Typical examples are coral snakes (*Micrurus* sp) and *Lachesis* sp in the Americas. Biochemical and toxinological analyses could lead to the identification of the most relevant toxins, which could be cloned and expressed in prokaryote or eukaryote systems. Immunization with cDNA encoding for relevant toxins has been explored at the experimental level with encouraging results [123–125]. Bioinformatic tools can be used for the selection of the most appropriate sequences [35].

Recombinant toxins could be used in animal immunization, thus eliminating the need of animals as source of venom. This alternative is useful for venoms whose toxicity depends on one or few toxins, such as the South American rattlesnake *Crotalus durissus*, whose toxicity is based primarily on the neuro- and myotoxic PLA$_2$ crotoxin and of a thrombin-like serine proteinase [126–128]. Immunization with these two toxins will likely generate an antivenom effective in the neutralization of the most relevant toxic effects of whole venom. Similarly, the toxicity of *Loxosceles* sp spider venom is mostly based on sphingomyielinase D. A recombinant enzyme has been generated to produce an antivenom effective in the neutralization of the crude venom [129]. Investigating further the proteomes of medically-relevant species of low abundance and difficult acquisition would allow the identification and cloning of their main toxins to generate recombinant proteins for immunization.

8.2. Monoclonal antibodies

Monoclonal antibodies (MAbs) have been successfully developed for therapeutic use in various clinical fields [130,131]. MAbs with neutralizing capacity against snake venom components have been generated for various types of toxins [132–138]. The potential use of this technology relies on successful experimental results [132]; chimeric or humanized versions of these MAbs may possibly reduce adverse reactions [139,140]. However, MAbs antivenoms are likely to have two main drawbacks: (a) the production cost of these antibodies is higher than that of polyclonal antibodies, thus reducing their accessibility to developing countries; (b) most snake venoms are highly complex mixtures of many different proteins [141,142], several exerting relevant toxic effects that should be neutralized by specific antibodies. In these cases, MAb-based antivenoms should probably include many MAbs specific for different toxins, an issue that complicates their production and licensing, and increases cost. Nevertheless, it remains to be evaluated whether MAbs could be of benefit against venoms of difficult

acquisition, with poorly-immunogenic toxins or whose toxicity depends on one or few proteins.

8.3. Recombinant and engineered antibodies

Combinatorial antibody generation using molecular cloning techniques represents a valuable alternative for antivenom generation. Recombinant single chain Fv antibodies (scFv) have been successfully generated against various snake venom toxins, and could neutralize toxic activities of some venom components [143–147]. A promising alternative is based on recombinant 'nanobodies', single domain antibodies comprised by the variable region of the heavy chain-only camelid IgG [148]. Encouraging advances have been made for the neutralization of scorpion venom toxins using nanobodies or scFv [149–151]. A bispecific construct against two different types of scorpion venom toxins was effective in abrogating lethality of the whole venom in mice even when administered late in the course of envenoming [149]. The potential use of these recombinant antibodies should be analyzed taking into account their pharmacokinetic profile and the toxicokinetics of venom proteins. In general, low molecular mass antibody fragments, such as Fab, scFV, or nanobodies, are rapidly eliminated by the kidney, thus having a reduced half-life [152,153]. Accordingly, the use of Fab antivenoms in the treatment of viperid snakebite envenomings has been associated with recurrence of envenoming several hours after antivenom administration, a consequence of their reduced half-life [83,153,154]. A similar scenario will likely occur in the case of scFv antibodies and nanobodies. However, for low molecular mass toxins of rapid absorption and high diffusibility, which rapidly reach their targets, such as elapid snake α-neurotoxins, these low molecular mass antibodies may bind toxins in the tissues more readily than IgG or F(ab')$_2$ fragments. It is important to investigate the potential therapeutic usefulness of these recombinant antibody fragments in diverse experimental models of snakebite envenoming to determine whether they could be effective in the neutralization of low molecular mass, rapidly acting toxins.

9. Quality control aspects

9.1. Physicochemical analyses

In-process and end-product quality control of antivenoms requires research and development efforts to further improve their quality, efficacy and safety. Since the physicochemical characteristics of antivenoms significantly influence their tolerability [46,58,89], incorporation of routine assays for physicochemical properties is necessary. In this regard, the quantification of turbidity and high molecular mass protein aggregates, by turbidimetric and chromatographic analyses, respectively, should be encouraged (see for example [88]). Such analyses are particularly relevant for freeze-dried preparations, as poorly-controlled freeze-drying generates protein aggregation and turbidity. Novel laboratory assays to quantify excipients used in antivenom manufacture or formulation are required. Likewise, validation of assays used in quality control laboratories should also be supported. Every laboratory should make efforts towards the validation of QC procedures, including those of well-established techniques such as the antivenom potency test [155].

9.2. Substitution of in vivo tests by in vitro assays

Assessment of antivenom potency still relies on traditional mouse lethality assay, the gold standard for ensuring the preclinical neutralizing efficacy of antivenoms [11]. Nevertheless, this assay leads to animal suffering due to the toxic effects (in particular pain) induced by venoms. Preclinical tests to assess the control of hemorrhage, myonecrosis, defibrination and edema also involves animal models [11,30]. The development of in vitro assays to substitute for animal models is a highly relevant task for future development of antivenom quality control as a way to achieve the 'three R' goals of animal experimentation, i.e. Reduce, Refine and Replace animal tests [156].

The biochemical and toxicological complexity of snake venoms greatly complicates the search for substitutive in vitro tests. Snakebite envenomings are complex pathophysiological processes often involving the simultaneous action of various types of toxins, thus representing a complex scenario that requires an integrative approach for a proper understanding [157]. In this context, the development of in vitro tests should ideally be based on a careful analysis of the predominant toxic proteins present in a particular venom, and on the understanding of the mechanisms by which these toxins exert their deleterious effects. A number of studies have succeeded at implementing in vitro tests giving a good correlation with the in vivo lethality assay. They include enzyme immunoassays [158–160], although some other enzyme immunoassays show a poor correlation with lethality tests [161,162]. For crude venoms, and since these assays detect antibodies against all venom components regardless of their role in toxicity, the reactivity against some highly immunogenic venom proteins, devoid of toxicity, may give high absorbance values not related with the neutralizing potency. Other studies have focused on the neutralization of venom activities, such as indirect hemolysis, i.e. phospholipase A$_2$ activity [163]. Some workers have implemented in vitro assays which assess neurotoxicity and myotoxicity in nerve-muscle preparations [164,165]. The use of cell culture systems to study cytotoxic venoms activity is an alternative for necrotizing venoms, e.g. from many viperid and some elapid species [166,167]. Fertile hen's eggs have also been used for assessing venom toxicity and antivenom potency, since despite being in vivo assays, these tests are performed at a stage in neurological development when pain sensation has not appeared yet [168,169].

The complexity and diversity of the pathophysiology of snakebite envenoming demand careful case by case analysis of medically-relevant venoms to design the most appropriate assays for assessing antivenom neutralizing ability. In some cases, the toxicity of a venom is based on a single toxin or a group of few toxins. This is likely the case of some elapid venoms [170] whose toxicity is based predominantly on the action of neurotoxins. On the other hand, the toxicity of some viperid venoms largely relies on the systemic action of P-III metalloproteinases, such as in the case of Echis ocellatus, which contain potent hemorrhagic and procoagulant metalloproteinases [171] and some Bothrops sp venoms [20,172]. When the key lethal toxin is identified, then the ability of an antivenom to react or neutralize this toxin is likely to correlate with the neutralization of lethality. A good example is crotoxin. This dimeric phospholipase A$_2$ is responsible for three of the most conspicuous clinical manifestations in envenomings by subspecies of the South American rattlesnake, i.e. neurotoxicity, systemic myotoxicity and renal failure [126,128]. Therefore, an immunoassay to detect antibodies against crotoxin, or a test designed to assess the neutralization of its phospholipase A$_2$ activity, are likely to correlate with the in vivo toxicity of this toxin and of the whole venom. In the case of venoms whose toxicity greatly depends on metalloproteinases, an immunoassay to detect antibody titer against the most abundant metalloproteinase, or a functional test to assess the neutralization of proteolytic activity of the enzyme, are likely to correlate with the neutralization of lethality. However, in many other cases, toxicity results from the combined action of different types of toxins acting synergistically. For instance, the lethality induced by the venoms of many viperid snakes seems to

depend on the action of several types of toxins, such as metal-loproteinases, phospholipases A$_2$ and serine proteinases [173]. This greatly complicates the development of *in vitro* tests substitutive to the mouse lethality assay.

The complexity of this issue underscores the relevance of performing biochemical, proteomic and toxicological characterizations of venoms of species that inflict a high toll of snakebites in the world and which are used as immunogens for antivenom production. International cooperative research efforts should be undertaken to have a complete characterization of these venoms, some of which have already been studied in detail. With this information at hand, it would be possible to design *in vitro* methods to assess the neutralization of these venoms by antivenoms. Although it is likely that the mouse lethality assay will continue to be the gold standard for the final quality control of the potency of antivenoms, adequately validated *in vitro* tests should be introduced for assessing antivenom antibody titre in immunized horses as well as for in-process quality control. This would greatly reduce the use and suffering of animals in antivenom production.

10. The need for pre-clinical and clinical assessments in antivenom development

As clearly stated in the WHO guidelines [11], the development of new antivenoms and the introduction of currently approved antivenoms to new regions demand careful pre-clinical and clinical assessment of efficacy and safety. A series of laboratory tests has been developed to determine the neutralizing capacity of antivenoms against the most relevant toxic activities of snake venoms [11]. Collaborative research projects are mandatory to properly assess the pre-clinical efficacy of currently available antivenoms and of new antivenoms, as has been done in Latin America [32]. There is a crucial need to develop local capacity in many countries to collect venom from indigenous snakes in order to prepare pools for use not only for the manufacture of antivenoms, either locally or abroad, but also for pre-clinical assessment of the efficacy of antivenoms distributed in those countries. On the other hand, the introduction of new antivenoms for clinical use requires a meticulous clinical assessment of efficacy and safety through well-designed clinical trials [11]. This arena of pre-clinical and clinical testing of antivenoms should be based on international collaborative efforts that take advantage of the expertise developed by a number of groups in these fields.

11. Concluding remarks: the need to promote innovation in the context of international networking and cooperation

A number of areas demanding technological innovation in antivenom production and quality control have been highlighted in this work. Antivenom production is largely within the scope of public and private laboratories in developing countries, and antivenoms are largely distributed and used in countries of Africa, Asia, Latin America and Oceania. Consequently, careful consideration should be given to the economic aspects of antivenom manufacture and marketing, since their price should remain affordable to patients and public health systems in developing countries. Therefore, a careful cost-benefit analysis has to be done when introducing new manufacturing steps. Innovation programs aim at improving antivenoms on the basis of low cost interventions should be prioritised.

Future innovation in antivenom production and quality control should include concerted efforts on taxonomy, epidemiology and clinical research, proteomics and transcriptomics, pharmacology, immunology, veterinary medicine, plasma fractionation technologies, process engineering, and quality control tests, among others (Figs. 1–3). Such complex disciplinary landscape necessarily requires the collaboration of groups working in different areas and different countries/regions. In particular, well-established and emerging production laboratories located in developing countries need to promote links with research and production groups in both developing and developed countries to circumvent their limitations and potentiate their strengths. The expertise gained by consolidated manufacturing groups should help less developed laboratories through a number of mechanisms involving technology transfer programs, training activities, exchanges, and workshops, using information and communication technologies to foster these interactions, along the growing networking trend in many scientific and technological fields [174]. Partnerships of different sorts, involving both south–south and south–north cooperation, and the participation of the WHO and non-governmental groups, like the Global Snakebite Initiative [175] and international foundations, should play a leading role in promoting these initiatives. Likewise, the open access to novel developments within this field should be encouraged. In summary, an active process of innovation and development of antivenoms needs to be encouraged, in a frame of international coordination, cooperation and networking. In the long term, these efforts, together with other actions in the public health realm, will greatly contribute to alleviate the burden of human suffering provoked by snakebite envenoming.

Acknowledgements

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