REVIEW / ARTÍCULO DE REVISIÓN

Current situation of snakebites envenomation in the Neotropics: Biotechnology, a versatile tool in the production of antivenoms

Elizabeth Romo[1], Marbel Torres[1,2,3], Sarah Martin-Solano[1,2,4*]

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[1] Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y la Agricultura, Universidad de las Fuerzas Armadas-ESPE, Sangolquí, Ecuador.
[2] Grupo de Investigación en Sanidad Animal y Humana (GISAH), Carrera de Ingeniería en Biotecnología, Departamento de Ciencias de la Vida y la Agricultura, Universidad de las Fuerzas Armadas-ESPE, Sangolquí P.O. Box 171-5-231, Ecuador.
[3] Immunology and Virology Laboratory, Nanoscience and Nanotechnology Center, Universidad de las Fuerzas Armadas, ESPE, Sangolquí, Ecuador.
[4] Grupo de Investigación en Biodiversidad, Zoonosis y Salud Pública, Universidad Central del Ecuador.
Corresponding author: ssmartin@espe.edu.ec

Abstract: Snakebite envenomation is a neglected tropical disease that affects millions of people around the world with a great impact on health and the economy. Unfortunately, public health programs do not include this kind of disease as a priority in their social programs. Cases of snakebite envenomations in the Neotropics are inaccurate due to inadequate disease management from medical records to the choice of treatments. Victims of snakebite envenomation are primarily found in impoverished agricultural areas where remote conditions limit the availability of antivenom. Antivenom serum is the only Food and Drug Administration-approved treatment used up to date. However, it has several disadvantages in terms of safety and effectiveness. This review provides a comprehensive insight dealing with the current epidemiological status of snakebites in the Neotropics and technologies employed in antivenom production. Also, modern biotechnological tools such as transcriptomic, proteomic, immunogenic, high-density peptide microarray and epitope mapping are highlighted for producing new-generation antivenom sera. These results allow us to propose strategic solutions in the Public Health Sector for managing this disease.

Key words: Antivenom, biotechnology, neglected tropical disease, omics, recombinant antibody.

Introduction

Neglected diseases occur in tropical and subtropical climates, specifically in rural areas where access to clean water, sanitary conditions, and medical care are limited[1]. They are caused by various pathogens such as viruses, fungi, bacteria, parasites and toxins, causing health, economic and social consequences. The term neglected is because these diseases are absent in most public health programs[2] affecting many people generating disability[3] and unemployment[4]. In addition, these diseases cause significant effects on the economy of developing countries due to the high cost of the treatments[5,6].

The World Health Organization WHO, in its NTDs portfolio, included 17 snake-caused diseases. Snakebite diseases were previously not on the NTD list, but since 2017, public health strategies have been planned for prevention, control and treatment[7]. Snakebites envenomation is found in Latin America, Africa, Asia and Oceania, poor rural tropics populations[8-11]. These countries have an absence of public health policies[12] pointed to snakebites diseases, so; they have no access to health services[1,13] and have a shortage of both medical supplies and trained human medical equipment.

More than 4 000 snake species worldwide, but only 250 are known of medical relevance[2]. Regions with the most incredible diversity of venomous snakes include Latin America and Asia[12]. Snakebite should be considered an NTD priority because it involves a wide snake diversity of species and, thus, a variety of toxins[14]. Snakes causing most ophidian accidents belong to the Viperidae and Elapidae families and the genera *Bothrops* and *Micrurus*[5,15,16]. Several risk factors, such as climate[17] and ecology[18], predispose to increased ophidian accidents[19]. Both rainy seasons and snake abundance5 cause a higher snakebites incidence[5,20,21].

Generally, global data for snakebites are not accurate, showing variability mainly due to scarce and not representative epidemiological studies[5,19,21]. Most hospital reports[22] and surveys[12] do not report important data such as incidence, mortality, and physical and psychological consequences suffered by patients[23]. According to Pach *et al.*[21], five million snakebites are reported annually worldwide, two to three million results in poisonings and 80 000 to 130 000 people die from these diseases. In Latin America and the Caribbean, hospital reports indicate approximately 70 000 cases of snakebites per year, which may be underestimated[24,25].

As a mega-diverse country, Ecuador has 40 poisonous snake species[22], of which 17 are responsible for 99% of poisoning cases. The most significant number of patients are found in the Amazon region, followed by the coastal area and the Andean region[26], which correlates with one of the studies of the geographic pattern of poisonous snakes[22]. Among the toxic snake families, the most representative is *Viperidae* and *Elapidae*[23,26].

Poisons are a set of proteins, peptides and enzymes that cause toxic effects[20] in the pathology of snakebite envenomation. The toxic profiles of each venom vary according to the geographic location and snake taxonomy[27,28], generating a wide range of local and systemic pathologies, inclu-

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ding blindness[29,30], necrosis[31,32], paralysis[33], respiratory[34,35], renal[36,37] and cardiac insufficiency[38]. The only specific treatment currently available is antivenom (Table 1) or antisera since its development in 1894[39,40]. The traditional method for producing antivenoms is based on animal hyperimmunization with non-lethal venom doses with the following collection of large amounts of plasma[11,41].

Almost all countries in the world have limited antivenom availability[1,13,42,43] due to low interest in drug research and development, costs, safety, efficacy, and inefficient antivenom distribution[5,13,21,23]. However, several investigations are carried out with different types of antibodies[44,45], using pharmacological molecules[46,47] and innovative DNA immunization strategies[48,49] to inhibit or reduce toxic effects.

This review aims to update the real situation of snakebites in the Neotropics from the epidemiological point of view and also to expose current biotechnological tools that could be implemented in the future to solve the drawbacks in the production and availability of antivenoms.

Snakebite in the Neotropics: Ecological and Environmental Aspects

Ecosystem alteration, agriculture and environmental conditions modify the ecological patterns in the geographic distribution of the different snake species[59]. The highest incidence rate is concentrated in rural areas; urban spaces report snakebite cases[60,61].

Venomous and non-venomous snakebites are the result of the interaction between humans and various snake species found in specific habitats such as jungles[62], forests[63,64], arid[65,66] and urban areas[67,68]. Snakes, humans and the environment are the three components of this ecological interaction where one part can influence the other one while also affecting the third one. A circular dynamic characterizes this interaction, so it is necessary to know and relate these components. According to Guedes *et al.*[69], 659 snake species were recorded in the Neotropics, where species richness and phylogenetic diversity are mainly concentrated in the Amazon region of Brazil, the Andean region of Colombia, Ecuador and Peru, and some Central America regions. On the other hand, few species are recorded in the Caribbean. In the Neotropics, the most abundant species are *L. muta*, *Micrurus frontalis*, *B. jararaca*, and *Bothrops erythromelas*[69]. These snakes are of medical and clinical importance in the Neotropics and belong to the *Colubridae*, *Elapidae* and *Viperidae* families[70].

Agriculture causes geographic and ecological change in ecosystems, as forests are replaced by cultivated croplands[71]. Humans have been engaged in agricultural activities such as planting crops and raising animals since the Paleolithic era (12 000 to 5 000 years BP), which continued over the millennia to the present day[72-74]. Many people do this job and find themselves vulnerable to venomous snakebites[75-77]. According to Suazo-Ortuño *et al.*[78] research on agricultural conversion, snakes are not susceptible to changes in their habitat; the species diversity in agricultural areas is not diminished, and snakebites risk is maintained. Several epidemiological studies determined a higher incidence in tropical rural areas where farming and grazing activities are carried out under unfavorable conditions[8,14,19].

The most significant number of cases are recorded in rainy seasons associated with natural phenomena such as floods, hurricanes and cyclones[79]. In the Neotropics, the El Niño phenomenon causes heavy rains that increase the incidence of snakebites[80]. Snakes are ectoderm animals; temperature increases cause snakes to migrate to more temperate zones that are inhabited by humans[81]. Climate change then causes a geographic redistribution of snake species due to alterations in environmental temperature[82-84].

Continent	Snake species	Treatment
Australia[50,51]	*Pseudonaja* spp. *Oxyuranus* spp. *Notechis scutatus* *Tropidechis carinatus* *Austrelaps* spp. *Hoplocephalus* spp.	Monovalent antivenom Polyvalente antivenom
Asia[52]	*Naja* spp. *Bungarus* spp. *Daboia* spp. *Echis* spp. *Trimeresurus* spp.	Monovalent antivenom Polyvalente antivenom
United States[53] **and Canada**[54]	*Crotalus* spp. *Agkistrodon* spp. *Sistrurus* spp. *Micrurus* spp.	Polyvalente antivenom
Latin America[11,55]	*Bothrops* spp. *Crotalus* spp. *Lachesis* spp. *Micrurus* spp.	Polyvalente antivenom
Africa[56,57]	*Echis* spp. *Bitis* spp. *Naja* spp. *Dendroaspis* spp.	Polyvalente antivenom Monovalent antivenom
Europe[58]	*Vipera berus* *V. aspis* *V. ammodytes*	Monovalent antivenom

Table 1. Treatment for snakebite envenomation around the world.

Clinical findings, Social, and Economic Impact of Snakebite Envenomation

Venomous snakes can cause several local and systemic pathologies such as hemorrhage[85,86], necrosis[87,88] and renal failure[89,90]. Complications result in physical and psychological injuries[91,92] in the short and long term, which can have an economic impact, including death.

Short-term sequelae occur immediately after the snakebite but can be controlled in a reasonable time. Among them, local hemorrhage[93], anemia[94], edema[95], abscesses[88] and bacterial infections[96,97] are found. If these complications are not treated promptly and adequately, they can lead to systemic complications that can result in disability[98].

Long-term sequelae appear approximately six months after snakebite envenomation and may persist for months or even years[92,99]. These sequelae are of the physical and psychological type, although the psychological effects have a late onset. For both, there is no follow-up for adequate treatment[100]. Among the physical sequelae, the most common are tissue injuries. Tissue necrotization can trigger compartment syndrome[101], which must be addressed by surgical treatment. This procedure causes loss of tissue and skeletal muscle function[3]. In some cases, amputations must be performed that generate permanent disabilities[102]. The renal dysfunction developed in patients affected by ophidian accidents can be persistent and progress to acute and chronic renal dysfunction that strictly requires dialysis treatment[103-105].

Psychological sequelae do not derive from the toxic effects of the envenomation. Still, they are the result of the traumatic process of the snakebite in which the patient suffers from the physical and economic consequences of the ophidian accident. Depression and post-traumatic disorder are the most reported effects affecting 25-45% and 43% of the patients evaluated, respectively, being the leading causes of morbidity[106,107]. Less common psychological effects are headaches, vertigo, hysteria, and delirium, but cognitive functions are not yet determined to be affected[91,99]. Long-term psychological sequelae also cause deterioration in the family and educational context. Patients present negative attitudes that prevent them from continuing their work, generating an economic and social impact[107-109]. Timely treatment would improve the life quality of snakebite victims. A first aid intervention, cognitive and behavioral psychotherapy allows for to reduction of psychological and psychiatric symptoms[92,110].

In addition to the physical and psychological sequelae, the economic consequences are aggravated by the absence of rehabilitation after the ophitic accident. This fact hinders labor insertion. Most of the time, the rehabilitation cost must be assumed by the patient itself, who usually does not have the resources to do so. The family economy in areas with a high rate of snakebites is categorized as impoverished rural areas[12,111].

Agricultural activity is the livelihood of these communities, being limited by the economic expense of the sequelae they suffer. According to these situations, conditions for a worthy life are reduced[112-114]. Finally, the economic impact affects the nuclear family and negatively affects local and national productivity[115].

Unfortunately, some case studies do not detail the patient's conditions under which they die due to snakebites[116]. This fact makes it difficult to show how high a priority this disease is. Lizarazo *et al.*[117] reported a case of a farmer who suffered a snakebite caused by the *B. asper* snake that produced a cerebral hemorrhage. Unfortunately, the antivenom administration was late; he presented a multiorgan failure and died. Hospital reports are vital in managing this neglected disease, so standardized processes should be implemented in public health centers.

Global and Regional Snakebites Burden

Global and regional snakebite envenomation burden has not been accurately determined because of scarce information and studies estimating the incidence and mortality of SBE[118]. Existing data are based on hospitals' epidemiological reports that do not provide evidence of the true SBE burden[24]. Collecting information on snakebites and envenomations is difficult because most victims live in rural and remote areas with limited access to health services[55]. In addition, people in rural communities prefer to treat themselves with traditional methods and do not go to hospitals[1].

In 1954 an estimated 500 000 poisonings were estimated[119], and in 1998 the estimate increased to 5 million snakebites per year[1,120]. According to Gutiérrez *et al.*[121], the snakebite burden has an estimated 2.5 million bites per year, concentrated in South Asia, sub-Saharan Africa and Latin America. Through a regional comparison, it has been possible to determine the global level and the regions most affected by snakebite envenomation. The annual envenomation cases vary from region to region: Europe, where non-venomous snakes mainly cause snakebites, reported 8000 cases; North America 5 000 to 10 000 patients; the Middle East 15 000 to 40 000, Africa 43 000 to 1 000 000, Asia 121 000 to 2 000 000, Australia 10 000, Oceania 10 000 to 500 000 and Latin America 60 000 to 300 000[1,120-122] respectively.

Snakebite occurs in different geographical environments, whose social, economic and ecological factors may be similar, allowing the development of social and technological strategies to cope with this disease in the context of public health. Gutiérrez *et al.*[123] evaluated the snakebite envenomation situation in Costa Rica, Nigeria and Sri Lanka, the most affected regions worldwide. The annual SBE burden is similar in Nigeria, with 43 000 reported cases, and in Sri Lanka, 40 000 reported cases, while in Costa Rica, case reports were much lower, reporting just only 500 cases.

The scientific community in Neotropical countries has conducted several epidemiological studies. The results may overlap with other existing ones, and some countries do not publish any information[24]. In the Neotropics, the countries with the most scientific publications on snakebite envenomation are Costa Rica, Colombia, Ecuador, Argentina and French Guiana[1].

The lack of data reliability and accuracy is due to deficient information systems in the different countries and because victims from rural areas prefer to use traditional treatment methods based on medicinal plants[124,125]. In Latin America, Chippaux[5] reported around 60 000 cases between 2014 and 2016 of snakebites per year and just 370 deaths. In another study, Kasturiratne *et al.*[1] made epidemiological estimates of SBE obtaining 115 000 cases of snakebites and 2 000 deaths from 1985 to 2007.

Variations in epidemiological indicators such as burden, incidence, prevalence and mortality are due to the influence of environmental and anthropogenic factors. Also, the period and geographical area of epidemiological evaluation and the El Niño current have different effects according to geographical location[126]. The countries with the highest incidence of around 100 000 cases of SBE per inhabitant

in the Neotropics, according to the official health reports of each country, are Panama with 55.8, French Guiana with 21.1, Venezuela with 18.9, Costa Rica with 15.0 and Brazil with 13.4[5]. Values of SBE burden vary among scientific publications and may be over or underestimated. The annual incidence of snakebites worldwide is about 6.2 per 100,000 inhabitants, while mortality is 0.04 per 100,000 inhabitants[5].

Antivenom Production: Current Status in the Neotropics

Antivenom production in the Neotropics dates back to the beginning of the 20th century at the Butantan Institute in Brazil in 1901, considered one of the pioneering laboratories in the region[118,127] together with the Clodomiro Picado Institute in Costa Rica, founded in 1970[128]. In 2014, countries such as Mexico, Costa Rica, Venezuela, Colombia, Ecuador, Peru, Bolivia, Brazil and Argentina were antivenom producers with laboratories in Public Institutions[129,130]. Brazil, Mexico and Costa Rica were able to satisfy the antivenom demand at the national level and even cover the regional and global market[118,131-134].

In other cases, where antivenom needs are not supplied, it must be imported from other countries in the region and even from other continents, as in the case of Martinique and Saint Lucia, Caribbean islands, which import antivenom from France and the United States, respectively[118,135]. In the case of Ecuador, antivenom production was local at the former antivenom producer "Instituto de Higiene y Medicina Tropical, Leopoldo Izquieta Perez", which operated up to 2012 and was closed due to deficiencies in the production processes[13]. At the beginning of 2022, the National Institute of Public Health Research INSPI signed a cooperative agreement with the Regional Amazonic University IKIAM to implement a research project to optimize the experimental production of effective antiophidic sera in Ecuador. This project promotes a change in public health perception and in the snakebite envenomation victims' lives.

In addition to the importance of producing antivenoms to reduce the SBE burden, they should be validated through pre-clinical and clinical trials to determine their effectiveness against various venoms. This becomes in an essential task due to the diversity of snake species in the Neotropics. Several clinical studies have been conducted with antivenoms from the region to treat endemic snake envenomation136 and have successfully reduced the envenomation signs developed in the victims[137-139]. The results of these assays indicate the existence of cross-reactivity[106,130,133,140,141]among antivenoms and toxins affecting patients, while others cannot neutralize heterologous venoms[142]. Antivenoms produced commercially in the Neotropics at a laboratory scale are mostly derived from equine serum and neutralize venoms of the genus: *Bothrops*[143-145], *Crotalus*[146-148], *Micrurus*[149-151], *Lachesis*[140,152].

Currently, there are only three antivenoms approved by the Food and Drug Administration FDA for exclusive use in the United States: Antivenin® Wyeth[153], CroFab®[154] and Anavip®[155]. In the Neotropics, only the antivenom Antivipmyn®, manufactured by Bioclon Laboratories of Mexico, was recognized by the FDA as an orphan drug[156]. These orphan drugs are intended to treat diseases affecting a small number of people, less than 200,000[157]. However, the FDA-approved drugs derived from snake venoms such as Captopril and Batroxobin[158], obtained from the venom of *B. jararaca*, and the latter from *B. moojeni* and *B. atrox*[32,159]. These drugs have proved biomedical applications.

The efforts of antivenom research and production are evident in the published scientific literature; however, there are drawbacks related to the heterogeneity in the used production technologies, the quality and innovation of the pharmaceutical products obtained and the production volumes[129,160,161]. These variables cannot be analyzed due to the absence of updated and reliable epidemiological information that would allow having a base-line and thus supply the antivenom requirements. In addition, antivenom production in Latin America during 2020 was reduced as a consequence of the COVID-19 pandemic caused by SARS-CoV-2. Medical supplies and research were mainly focused on developing therapies and diagnostic kits to cope with COVID-19 health emergencies[162].

Snakebite in Ecuador

Epidemiological studies in Ecuador are limited[62,163]. No data has existed in the country's health system records in the last three years. From 2014 to 2018, the Public Health Ministry of Ecuador, through the Epidemiological Gazette, recorded 7 714 cases of snakebites in the country, with an average incidence of 9.37 / 100 000 inhabitants and an annual incidence of 9.0, 11.3, 10.4, 8.6 and 7.6 respectively[164]. In the Chippaux[5] study, incidence and mortality rates of 9.5 and 0.057 cases per 100,000 inhabitants were estimated in 2014-2015. In another study, Ecuador reported 9.8 cases per 100,000 inhabitants that resulted in 0.06 deaths per 100,000 inhabitants each year in 1998-2007[80]. The annual incidence of snakebites in Ecuador does not have significant variations even though the Spatiotemporal analysis differs, 11.5 /100 000 inhabitants[80],7.7-11/100 000 inhabitants[165] and 9.5 / 100 000 inhabitants[5].

Morona Santiago and Manabí are the provinces that register the highest number of snakebite cases. However, it is observed that in both the coastal and Amazonic regions were found a more significant number of cases[164]. Snakebites are distributed geographically in the Coastal region (56-58%), Andean region (5-33%) and Amazonic region (11-37%)[80]. Studies conducted in indigenous communities indicate that the highest incidence of snakebites occurs in the Amazon region[142,166-168], associated with the distribution of snake species in the country[22,26]. Among the species causing snakebites in the country is the genus: *Bothrops, Bothriopsis, Lachesis* y *Micrurus*[79] and the species: *B. atrox, B. asper, L. muta, B. bilineata* y *Bothrops lojanus*[26,168]. Information obtained from available epidemiological studies indicates that those mainly affected are agricultural workers in rural areas[80]. Heavy rains such as the El Niño Phenomenon in January to June increase the incidence of snakebite cases throughout the country[80].

Biotechnological Approach to Snakebite Therapy

Drawbacks of available Antivenoms

Antivenom therapy is the most widely used therapy to treat several pathologies, including snakebite envenomation, by neutralizing the venom proteins[169]. Traditional antivenom production has several drawbacks affecting their safety and efficacy, such as the venom complexity, adverse reactions and production costs. Venom composition varies by snake diversity[69], geographic distribution[170,171], ontogenetic[172] variations and snake growth stage[173]. Toxins that make up snake venom have a great molecular and biological diversity, including protein and peptide content; non-protein

components such as carbohydrates, lipids, amino acids, nucleosides, amines and metal ions[174]. The main molecules of medical importance are grouped into dominant and secondary proteins: phospholipases, metalloproteinases, serine proteinases, three-finger toxins, hyaluronidases, and myotoxins, among others (Table 2)[175]. These factors limit the antivenom's neutralization spectrum, although several studies in the Neotropics indicate a good neutralization capacity of heterologous venoms, as mentioned in the previous sections.

Antivenoms are obtained from animal immunization, such as horses[184,185], donkeys[186], and sheep[187,188], hence the name heterologous antivenoms[189,190]. Antivenoms currently present several drawbacks, as 59% of patients treated with this therapy develop adverse events and side effects[191]. Early anaphylactic reactions cause headaches, vomiting, fever, and urticaria[192,193] and may or may not be an IgE-mediated response[194]. Antivenom can be composed of complete IgG, antibody fractions (Fab or (Fab')2) and other serum proteins of animal origin that can also cause adverse reactions. In addition, many antibodies do not neutralize the target antigen[195]. Different types of antibodies are involved in developing late anaphylactic adverse reactions, such as human anti-horse antibodies named heterophile. These antibodies form an immune complex deposited in the target tissues causing inflammation known as serum sickness[196,197].

Purification and enzymatic digestion techniques are being employed to improve the quality and safety of the drug product while reducing the effects of adverse reactions[198]. Antivenom formulations with antibody fragments maintain neutralizing capacity and minimize adverse effects[137,199]. This antibody-based production technique currently employed has several disadvantages due to the costs of animal maintenance and antibody purification techniques. These facts limit the reproducibility of this technology[14,189].

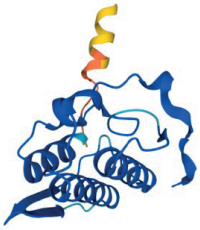
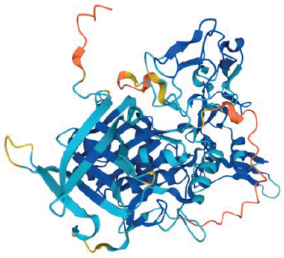
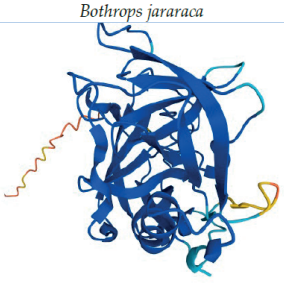
Toxins	Physiologic effects	3D-Structure
Phospholipases (PLA2s)[176,177]	Neurotoxic and myotoxic effects Severe necrosis Paralysis due to blockage	 UniprotKB: P24605 Myotoxin II *Bothrops asper*
Snake Venom Metalloproteinases (SVMPs)[178,179]	Systemic lethal hemorrhage Edema Hyperalgesia Inflammatory pain	 UniprotKB: P30431 HF2-proteinase *Bothrops jararaca*
Snake Venom Serine Proteinases (SVSPs)[180,181]	Alteration of hemostasis Edema Hyperalgesia External and internal bleeding	 UniprotKB: Q9PTU8 Bothrops protease A (BPA) *Bothrops jararaca*
Three-Finger Toxins (3FTXs)[182,183]	Neurotoxicity Hemotoxicity Flaccid paralysis Necrosis	 UniprotKB: C0HJR1 Micrurotoxin 1 *Micrurus mipartitus*

Table 2. Main snake venom toxins, physiological effects and its 3D-structure.

Based on this background, we expose the use of biotechnological tools with an innovative approach to improve the neutralization of venoms, increasing their efficacy and improving production yields. It is essential to consider biotechnology as a solution to the shortage of antivenoms in Latin America and the world since production costs can be reduced, and the pharmaceutical market in the production of antivenoms will be empowered.

Recombinant Antibody Technology

The first recombinant antibodies were developed by Georges Kohler and Cesar Milstein in 1975 using the hybridoma technology[200]. Recombinant antibody technology since then has become a potential therapy for snakebite envenomation. They are more effective because of their neutralizing capacity and reduced side effects such as anaphylactic reactions compared to animal serum-based antivenoms[198]. Using this technology, several antibody formats have been obtained in chimeric and humanized versions, whose therapeutic use is approved by the FDA in treating several diseases[201,202].

The hybridoma technology, nevertheless, has several disadvantages. The main one is related to the development of human anti-mouse antibodies that cause allergic reactions and decrease the lifetime of the therapeutic antibody[203]; however, this is still the most widely used technique for antibody production. Production of antibodies involves an immunological process in which the antigen undergoes proteolytic degradation, so the derived antibodies will not distinguish the antigen in its initial form[204]. Therefore, researchers work to find other technologies that solve these drawbacks and do not activate the complement system.

Antibodies for therapeutic purposes like IgG have a longer life and are more permeable[205]. Recombinant antibodies have various formats in the structure; they are assembled according to different combinations of heavy and light chains[206]. The single-chain variable fragment (scFv) and fragment antigen-binding[137] are the most commonly used formats because of their high affinity to the antigen, structural stability and shorter generation[207].

Currently, several studies demonstrate the efficacy and therapeutic potential in neutralizing various snake venoms using recombinant antibodies of multiple formats, e.g., camelids nanobodies against the poison of *B. atrox*[208] and *C. durissus terrificus*[209]; scFv against the venom of *Bothrops pauloensis*[210] and *L. muta*[211]. Recombinant antibodies, such as scFv and nanobodies, are FDA approved for treating the diseases, except for the treatment of snakebite envenomation[212]. In addition, the FDA and European Medicines Agency EMA has not approved the production of recombinant antibodies in *E. coli*[189].

The isolation of therapeutically effective antibodies has presented low yields due to the high purification costs using traditional methods[213-216]. Smith[217], in 1985 developed the phage display technique (PDT) that is independent of an immune system, making this technique the most selected since it does not generate immunogenicity in patients[218]. The PDT, as shown in Figure 1, consists of an *in vitro* phenotypic selection of antibodies expressed with the fusion proteins of M13 filamentous phages. At the same time, a genotypic selection is needed because the various genes encoding the antibodies of interest are found inside the phages[219,220]. The antibody selection and enrichment process are performed by affinity to the molecules of interest, in this case, the venom toxins[218,221]. This technology's advantages focus on controlling the process conditions such as antigen selection, immobilization, design of the antibody libraries, and binding and washing needs. In addition, it is a faster and low-cost technique compared to hybridomas production[204]. It is good to stand out that antibody production against snake venom toxins has only reached the laboratory scale; research is still ongoing for their optimization and further scaling up.

The next stage of affinity antibody selection is heterologous expression. There are several expression systems ranging from bacteria, yeast, insects, and plants to mammals, each with advantages and disadvantages[222]. Bacteria such as *Escherichia coli*[223,224] and *Bacillus subtilis*[225,226] have been employed as factories to produce heterologous proteins for therapeutic purposes because their genome is characterized and genetic manipulation is simple; they have rapid growth and bioprocesses have enabled large-scale production at low cost[167,227]. Castro *et al.*[228] produced a recombinant antibody, scFv, that neutralizes the BaP1 metalloprotein from *Bothrops asper* snake venom by expressing the antibody in a bacterial system using *Escherichia coli* as host (Figure 2). After protein extraction and purification, the yield was 280 ug of scFV per liter of bacterial culture. The drawbacks of these systems are due to the absence of post-translational modifications and a poor excretion system, as the stability of the proteins depends on the oxidative environment where it is secreted. In addition, inclusion bodies can be formed, hindering antibody purification[229-231]. Strategies to optimize antibody production in *E. coli* are listed in Table 3.

The most commonly used yeasts for these purposes are *Pichia pastoris*[240-242] and *Saccharomyces cerevisiae*[243,244]. Both are easy to grow, perform post-translational modifications such us disulfide-bonded and protein glycosylation, have a high growth rate and protein secretion levels are very high. Contrary to bacterial expression systems, protein secretion in yeasts constitutes a great productive advantage since the secreted proteins are harvested relatively quickly from the culture medium, so downstream processes are cheaper[242,245]. *Pichia pastoris* is also the most widely used yeast strain due to its ease of industrial scaling. It reduces costs and minimizes equipment used for implementing pilot or industrial bioreactors[246]. Yeast expression systems are used to produce recombinant antibodies and proteins with inhibitory action against venom toxins (Figure 2). The antimyotoxic protein DM64, which acts against phospholipases A2 of *Bothrops asper* venom[247], was successfully produced by a recombinant *Pichia pastoris*.

Mammalian cells are commonly used to produce biopharmaceuticals, antibodies and active protein[248]. Antibody production in mammalian cell systems is mainly selected by its ability to carry out post-translational modifications that maintain antibody stability so a correct protein function. However, expensive culture media due to nutrient requirements and high contamination rates limit this technology. In addition, yields are low and the slow production time increases costs[236,249].

Laustsen *et al.*[251] and Jenkins and Laustsen[250] estimated the cost of large-scale production of antibodies in the Chinese Hamster Ovary (CHO) cell expression systems using a fed-batch fermentation strategy. The production cost ranged from 20 to 250 USD for these pharmaceutical products. On the other hand, plasma-derived antivenom production is around a thousand dollars. Currently, no studies are estimating the cost of new screening and expression technologies applied to large-scale antivenom production.

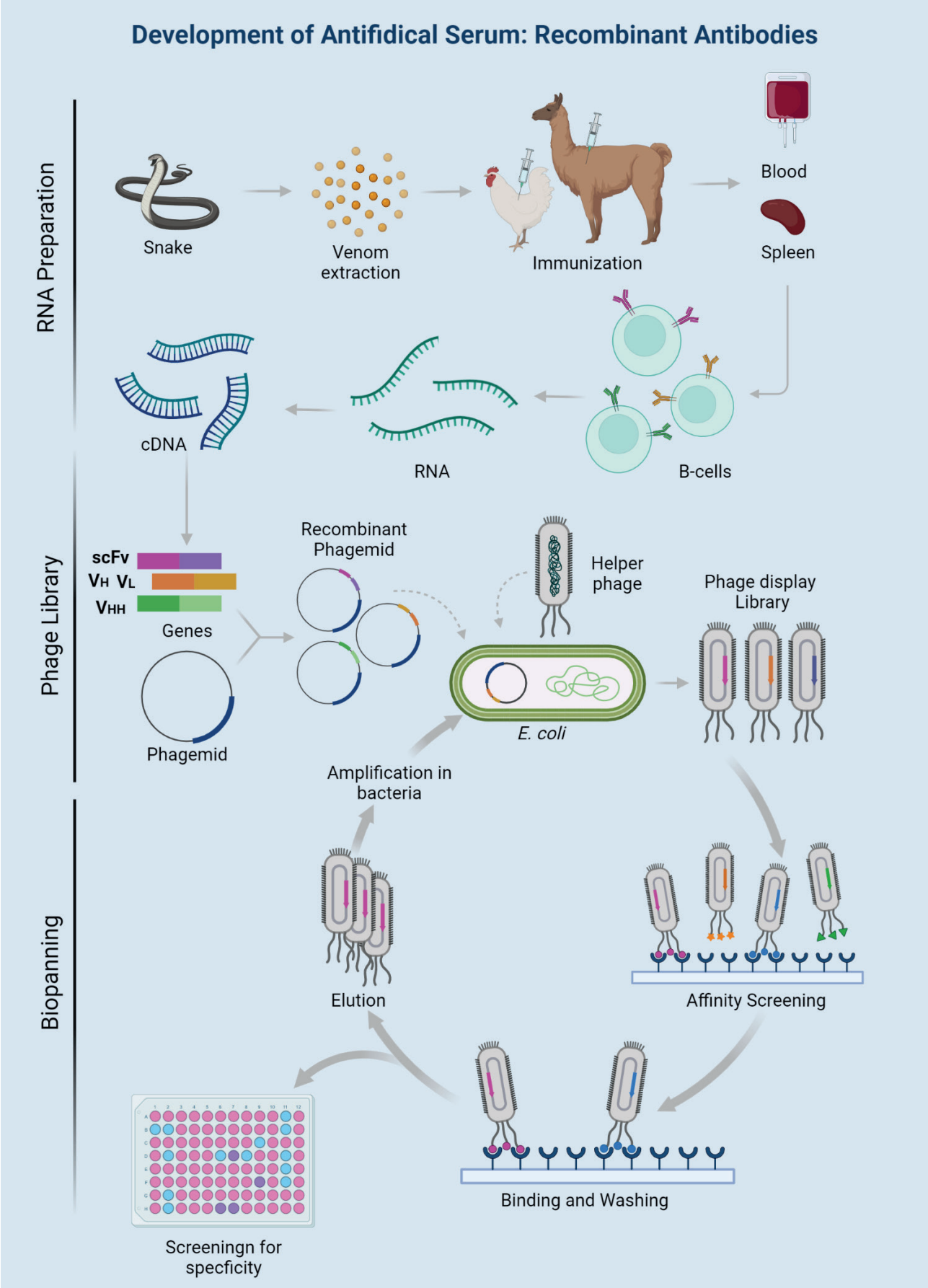


Figure 1. Phage display for selection of antibodies against snake antivenom. Image created using BioRender (https://biorender.com/).

Expression system	Advantages	Challenges
Periplasmic Expression[232-234]	• Correct protein folding by chaperones that catalyze the formation of disulfide bonds.	• Signal sequences are required, which affect production yields because they are unpredictable. • Low membrane transport performance. • Limited volume of periplasmic space.
Co-Chaperone Expression[235-237]	• Cytoplasmic expression of disulfide-rich proteins • It requires the expression of other chaperones that provide oxidative equivalents to generate de *novo* disulfide bonds.	• Low yields.
Inclusion Bodies[238,239]	• Protect against the potential toxicity of the expressed protein. • High performance and potential purities.	• Complex protein purification protocol. • Misfolded and inactive proteins.

Table 3. Strategies for the expression of disulfide-rich proteins in *E. coli*.

An alternative system to those described above includes insect cells advantageously as production hosts. This expression system can use chaperones for correct protein folding and own key metabolic pathways to carry out post-translational modifications, such as acetylation or glycosylation[251,252]. The system works with the baculovirus expression vector[253]. Insect cells are used as hosts to a greater extent for toxins production used as immunogens and for different *in vitro* toxicity assays. This technology is more complex but with high throughput and reproducibility at low costs[254].

Finally, we have plant-based expression systems. To date, several types of toxin antibodies have been produced experimentally[255-258]. Plants are considered biofactories because of the amount of biomass they generate, allowing large-scale production. They are low-cost and not susceptible to contamination. Nevertheless, even though the initial steps of N-glycosylation and N-glycan processing are highly conserved between plants, mammals and yeast, N-glycosylation patterns differ between them[259].

Antibody expression titers in plants are low, so approaches for expression improvement have pointed to expression cassette design, plant and tissue selection and plant material extraction techniques[260]. There are few studies of antibody expression in plants (Figure 2). One reported the extraction and purification from *Nicotiana tabacum* leaves of scFv against *B. pauloensis* venom[210].

Although different techniques currently produce monoclonal antibodies, other biotechnological alternatives can be employed to enable regional and global scale of antivenoms production.

Omics for the production of Antivenoms

Omics enable innovation in the health sector to broaden the understanding of physiological processes of pathologies involving various molecules such as nucleic acids and proteins[261] so, facilitating effective diagnosis and treatment[262]. Omics tools such as proteomics and transcriptomics are a fundamental axis in the design and production of antivenoms (Figure 3) as they are part of the preclinical evaluation and improvement of antivenom efficacy[170,263].

These technologies must comply with the good manufacturing practices detailed in the WHO Guidelines for the industrial production of antivenoms[43,264] to ensure the quality and safety of the pharmaceutical product[265]. Proteomic and transcriptomic guide researchers to understand the biochemical and toxicological variations in venoms to antivenoms[265] response. Omics tools should be included in antivenom production processes to validate the safety and quality of the bioproduct[266].

Transcriptomics

Transcriptomics studies genome-encoded RNA transcripts such as mRNA, rRNA, tRNA, miRNA, and non-coding RNA[267]. The mRNA is required for protein synthesis, and its abundance indicates the presence of a target gene. The transcriptome is subject to change due to time, environmental and physiological conditions[268]. Transcriptomics gives information on RNA diversity, transcriptional units, splicing mechanisms, post-transcriptional modifications and information of gene expression, regulation and signaling[267]. The transcriptomics workflow is depicted in Figure 3.

Transcriptomic studies of venoms and venom glands of some snakes from the Neotropics were carried out by Rodrigues *et al.*[269]. He compared the transcriptomic profiles of the venom and venom gland of *Bothrops pauloensis*, finding qualitative variations and low concordance with the proteomic profiles. Ontogenetic changes affect venom composition; in young species of *B. jararaca* there is a greater diversity of toxin precursors and elevated amounts of metalloproteinases compared to adult species[173]. The analysis of the ontogenetic factor is fundamental in the production of antivenom since the efficacy in neutralizing envenomations caused by juvenile species may be limited. Freitas-de-Sousa *et al.*[270] evaluated the environmental effect in captive and wild species of de *B. atrox*, the composition of the venoms does not present significant quantitative differences, thus, supporting the use of venoms from captive species for the production of the antivenom.

The use of transcriptomics as a tool for toxin discovery

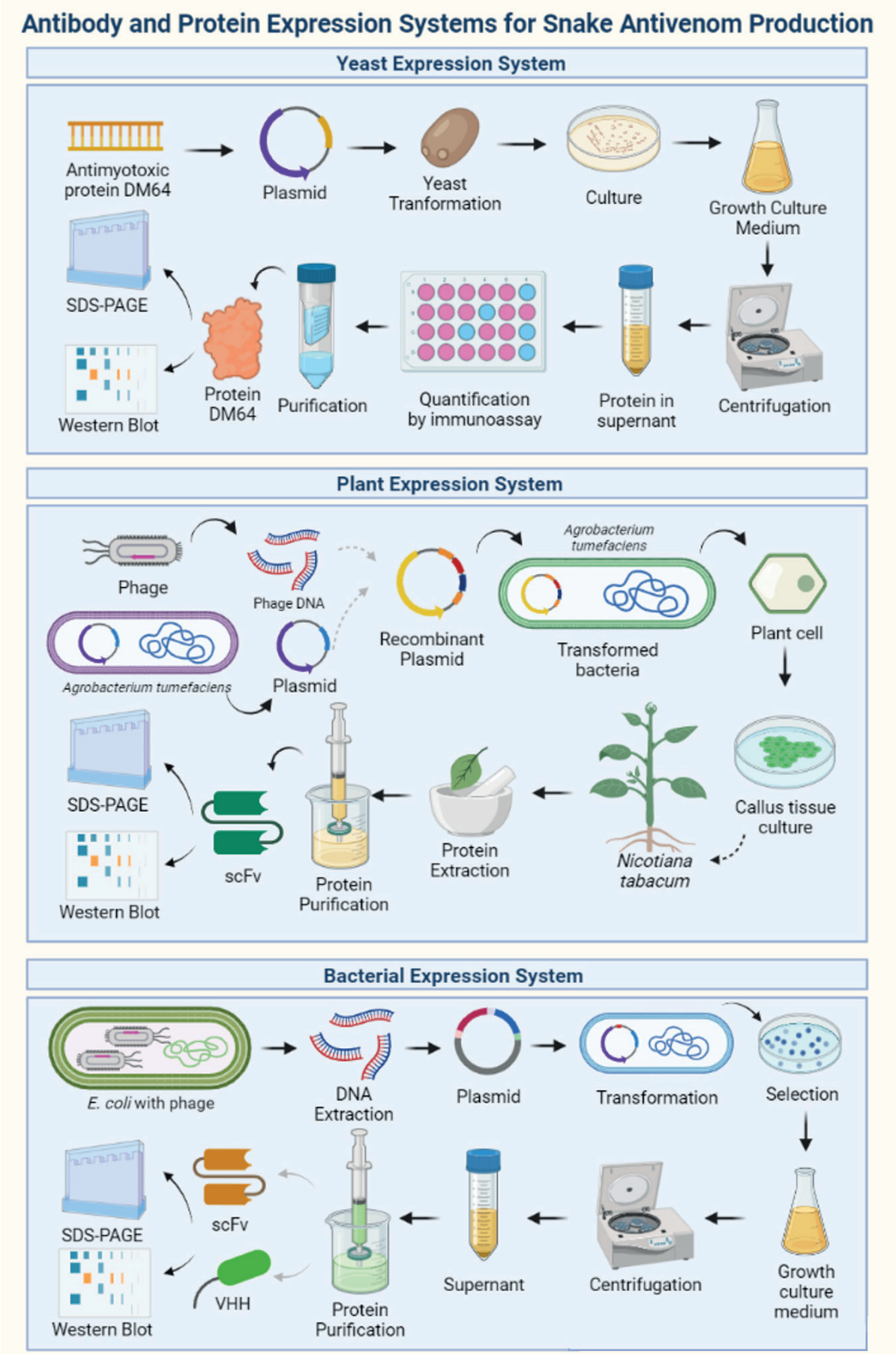


Figure 2. Protein and recombinant antibody expression systems for the production of snake antivenoms. Image created using BioRender (https://biorender.com/).

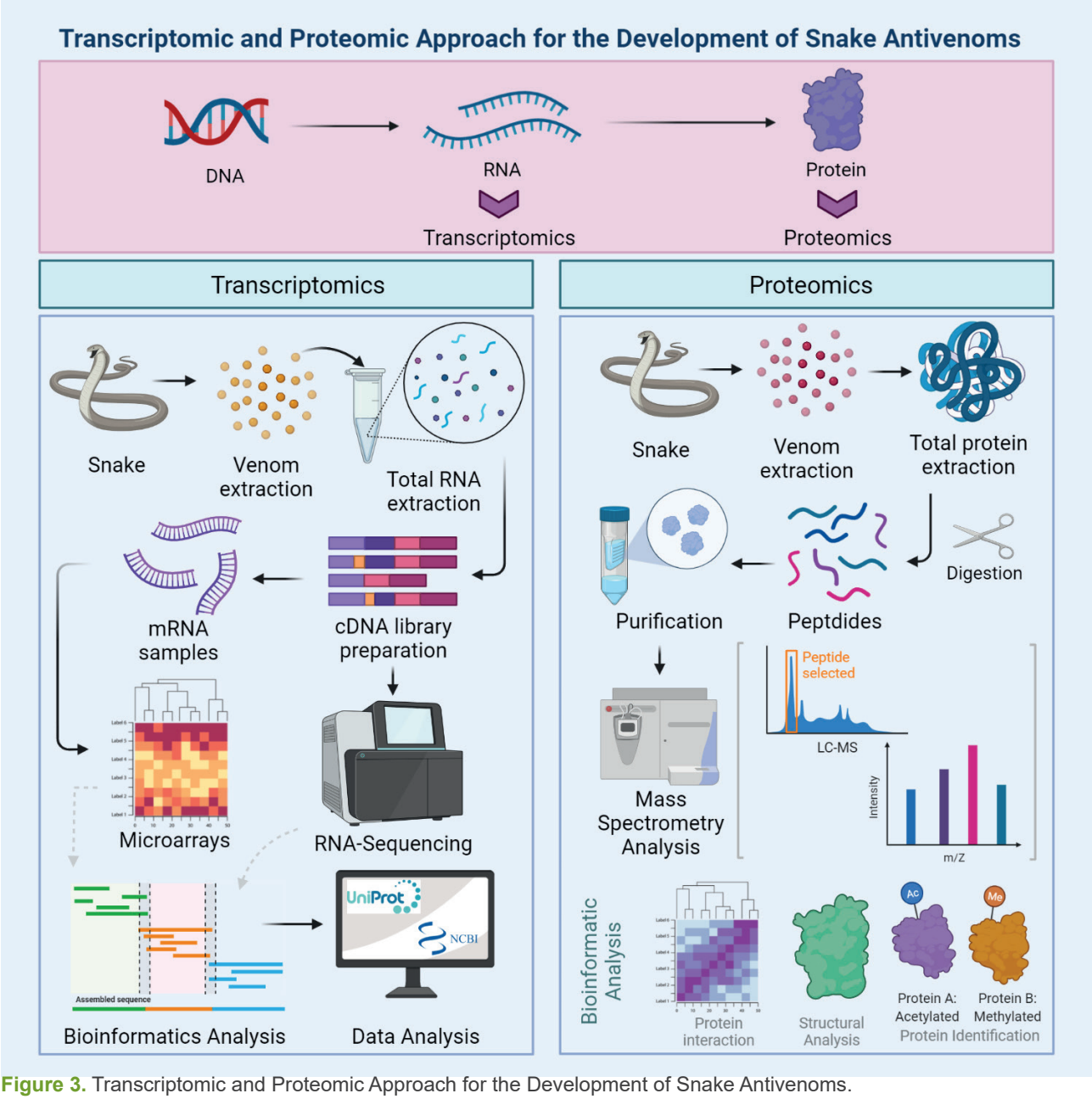


Figure 3. Transcriptomic and Proteomic Approach for the Development of Snake Antivenoms.

has displayed good results. In the venom gland of *Bothrops moojeni*, new toxins have been discovered, and amino acid sequences of unreported toxins have been obtained. These findings promise to know the function of new toxins and to design effective and neutralizing antivenoms[271]. Transcriptomics is used to know the complexity and composition of snake venoms and to evaluate toxins' immunogenicity at the molecular level, specificity and affinity for epitopes. The study of phospholipase A2 and three-finger toxin from *Micurus nigrocinctus* venom presented low immunogenicity[272].

Proteomics

Proteins are expressed in cells and perform cellular processes related to biological functions. Proteomics studies the entire set of proteins in a cell or organism[273]. It is characterized by being dynamic and influenced by time, space, environment and cellular modifications such as post-translational modifications[274,275].

Proteomics has several approaches to obtaining information about protein structure and functionality, including cellular expression, modifications, interactions and signaling[275]. The study of proteomics is essential if we consider proteins as gene products since proteins determine the phenotype. Genomics is static; the expression level of a gene will not always correlate with protein levels[276] due to post-transcriptional and post-translational modifications. The study of snake venom proteins is also known as venomics. Both venomics and peptidomics allow understanding of the biological processes in envenomation, development of new therapeutics and potential pharmacological applications of snake venom toxins[277]. Proteomics follows two main experimental approaches for its study: gel-based and mass spectrometry. The mass spectrometry approach is divided into two modalities: Bottom-up or Shotgun proteomics, where proteins undergo enzymatic digestion, and top-down analysis employs intact protein[278]. The proteomics workflow is depicted in Figure 3.

Venom proteomics studies showed variations in the

composition and functionality of toxins by several factors. A phylogeographic approach with proteomic tools has determined the venom phenotypes of snake species belonging to the Micrurus; the geographic distribution of venomous snakes and evolutionary mechanisms are very influential factors[279]. There are interspecific, intraspecific, qualitative and quantitative variabilities of snake venoms under different environmental conditions. Oliveira *et al.*[165] evaluated the proteomic profiles of 22 individuals of the *C. durissus terrificus*, new venom components were found with various enzymatic activities that cause other immunological and biochemical effects affecting antivenom production. Snake species, such as *B. atrox* and *B. jararaca*, develop a response to adapting environments that can produce several venoms-protein isoforms at the molecular level with different biological activities, complexity and enzymatic activity, which limits antivenom efficacy[280,281].

Immunogenomics

Immunogenomics is an essential tool for antivenoms development since epitopes mapping antibodies capable of recognizing these antigenic sites can be designed. This fact increases the neutralization capacity of antivenoms[282]. This tool, also known as antivenomics, allows identifying the recognition of certain immunogens by antibodies, a key factor in the clinical efficacy of antivenom in snakebite envenomation[283].

The antivenoms produced at Instituto Butantan and Instituto Clodomiro Picado present efficient neutralization of the venom of *B. atrox* and *Bothrops erythromelas* species in the northern region of South America and Brazil, respectively[170,284]. The commercial antivenom Antivipmyn Tri produced in Mexico by Instituto Bioclón exhibits immunoreactivity of *C. durissus cumanensis* venom in Colombia[285].

Toxins used to determine antibody responsiveness are also produced without the need for host cells. Protein synthesis is performed with the necessary components: dNTPs, amino acids, ATP, GTP, biological machinery that includes ribosomes, tRNA, RNA polymerases, initiation and elongation factors and a motor-like plasmids DNA carrying the correct information for transcription, translation and accurate folding *in vitro*. The main drawback of cell-free production is the low yield of proteins and their poor stability[286,287].

Bioinformatics, the best omics ally

The results obtained from these omics tools are massive, so computational tools are needed to facilitate data analysis[288,289]. Bioinformatics, through the use of algorithms and computational strategies, develops methods to analyze biological data, which include: data organization and curation, processing, annotation, statistical analysis, prediction and simulation[290]. The most commonly employed bioinformatics analyses are listed in Table 4.

Scientific evidence for decision-making in Public Health

The vast amount of information found in the published scientific literature on snakebite epidemiology, strategies and other associated issues can create confusion among decision-makers in governmental entities in the region and hinder the formulation of public health policies. Based on this systematic review, we propose actions that governments should implement according to their country's needs.

Start with creating a single national registry system to obtain a database on the epidemiology of snake bites. The information obtained will make it possible to know which groups are most affected. With this base information, strategic programs for prevention, control, monitoring, planning and research can be developed.

Follow-up programs for victims of venomous snakebites can reduce long-term sequelae. Prevention strategies should include educational programs and the provision of protective equipment in rural areas whose main activity is agriculture. Efforts should be made to strengthen the medical room for rapid action in snakebite emergencies.

Last but not least, governments should provide resources for the characterization of clinically meaningful snake venoms and promote research to create effective and low-cost diagnostic and therapeutic tools.

Conclusions

Snakebite disease is considered a neglected tropical disease due to its global, regional and national burden, as well as its social and economic impact on society. Although there are several prevention strategies and tools for treating this disease, a One Health approach is required because several actors are involved in its dynamics. Ecological, political, technological and medical aspects should be considered to allow us to manage and administer the correct registration of snakebite cases from public policies. It is essential to ensure the development of preventive programs and effective treatments for snakebite envenomation using current biotechnological tools for vulnerable populations. Preventive programs will improve the economic and social situation of the most affected regions today. The most modern biotechnological tools have been applied experimentally, but only on a laboratory scale, and the support of governmental entities is a crucial factor in enhancing the future industrial production and snakebite antivenoms scaled up.

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Conflicts of Interest

Authors do not claim any conflict of interest.

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Technology	Application	Supplementary techniques	Bioinformatics Analysis	Target Venom
Phage Display	Development of Antibodies, selection of scFv[210,211] and delection of VHHs [208,209].	Library construction Bio panning Sequencing ELISA Western Blot Colony PCR MALDI-TOF2	Multiple sequence alignment	*Bothrops atrox* *Bothrops pauloensis* *Crotalus durissus terrificus* *Lachesis muta*
Recombinant antibodies	Development of Antibodies, selection of scFv[210] and selection of VHHs[208,209].	Cloning PCR SDS-PAGE Western Blot Antibody expression and purification	N/D*	*B. atrox* *B. pauloensis* *C. d. terrificus*
Plant transformation	Production of recombinant antibodies[210]	Plasmid construction PCR Sequencing	N/D*	*B. pauloensis*
Proteomics	Venom phylogeny[279,291]. Identification of venom toxins[149,270,280,292]. Venom variability[165,170,284].	Protein, peptide and amino acid sequencing Liquid Chromatography: FPLC, RP-FPLC, RP-HPLC SDS-PAGE Mass spectrometric analysis: ESI , nESI-MS/MS, MALDI-ToF, MALDI-TOF-TOF, LC-MS/MS	Peptide sequence analysis	*B. atrox* *Bothrops jararaca* *Bothrops erythromelas* *Crotalus durissus collilineatus* *Micrurus ruatanus* *Ophryacus sphenophrys* *Porthidium lansbergii*
Peptidomics	Composition of snake venom[293].	Enzyme digestion LC-MS/MS	Peptide alignment	*B. atrox*
Transcriptomic	Identification of venom toxins[271]. Characterization of toxins[173,270]. Molecular basis of venom composition differences[173,269]. Antibody responses to toxins at the molecular level[272].	cDNA library construction PCR Sequencing Generation of ESTs High-throughput sequencing Next-generation sequencing	Assembly of contigs, sequence alignment, Dendogram (Neighbor-joining method), Protein modelling	*B. atrox* *Bothrops moojeni* *B. pauloensis* *B. jararaca* *Micrurus nigrocinctus*
Antivenomics	Efficacy of antivenoms[170]. Immunoreactivity[284,285].	Immunodepletion RP-HPLC SDS-PAGE Immunoaffinity assay	N/D*	*B. atrox* *B. erythromelas* *Crotalus durissus cumanesis*
High-density peptide microarray	Epitope mapping[294].	Library of peptides *in silico* Photolithographic synthesis Protein homology model	Multiple sequence alignment	*B. asper* *Crotalus simus* *Lachesis stenophrys*

Table 4. Current biotechnological technologies for producing snake antivenoms in the Neotropics use bioinformatics tools.

Bioinformatics	Identification of epitopes[295]. Phylogeny-based comparative analysis[291]	DNA extraction PCR Sanger Sequencing Protein sequence analysis Prediction of secondary protein structures Molecular modeling Peptides synthesis	Multiple sequence alignment Phylogenetic reconstruction (Bayesian inference)	*L. stenophrys* *P. lansbergii*
B-cell epitope mapping	Identification of peptides capable of inducing neutralizing antibodies[296-298]. Synthetic antigens for immunization protocols[298-300].	SPOT synthesis technique: peptides and genes Fmoc solid-phase synthesis MALDI-TOF ELISA Homology modelling	Sequence alignment	*B. atrox* *B. jararaca* *C. durissus* *Micrurus corallinus* *Micrurus frontalis*
Liposome encapsulation	Encapsulation of synthetic peptides for immunization[298,300].	N/D	N/D*	*B. atrox* *C. durissus*
Heterologous protein expression	Development of antitoxic protein as antivenom therapy[301].	Plasmid construction Transformant screening Protein expression Immunoassay Western Blot	N/D*	*B. asper*
Cell culture	*In vitro* alternative assay for antivenom pre-clinical evaluation[247].	Cell viability assay Neutralization assay Phase-contrast microscopy	N/D*	*B. jararaca*

*NO DATA

Table 4. Current biotechnological technologies for producing snake antivenoms in the Neotropics use bioinformatics tools.

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