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REVIEW

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Alternative methods for potency evaluation for *Bothrops* venom and antivenom: applications and perspectives for quality control

Métodos alternativos para determinação da potência de veneno e antiveneno botrópico: aplicações e perspectivas para o controle da qualidade

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ABSTRACT

Introduction: Accidents with venomous animals are classified as neglected tropical diseases and are currently the most frequent cause of intoxication in humans in Brazil. The only available treatment is the rapid administration of specific, quality-assured antivenoms. To ensure the efficacy and safety of these products, *in vivo* potency determination tests for venom and antivenom are performed during the production stages, until final release. Despite several studies on alternative methods to the murine assay, no method has been effectively validated. **Objective:** To compile alternative methods developed for *Bothrops* antivenoms, assessing the availability of the methods and the prospects and applications in *Bothrops* venom and antivenom production and quality control laboratories. **Method:** A search was conducted in PubMed, BVS, and Scopus databases between November 2021 and June 2022. 89 articles were identified, of which 31 were selected according to the eligibility criteria. **Results:** We observed in the alternative methods identified a preference of 42.80% of the studies for methodologies that use cell lines as an alternative method to the murine assays, and most of these works (58.30%) opted for a Vero cell line. **Conclusions:** Due to the diversity of toxins found in each genus of snakes, it is understood that the potency assay for antivenoms should be based on the evaluation and precise quantification of the inhibition of biological activity of venoms. Cytotoxicity assays are widely used and have been accumulating evidence of their suitability as an important alternative tool to the murine assay for quality control for *Bothrops* venom and antivenom.

KEYWORDS: Alternative Methods; Bothrops Venom; Bothrops Antivenom; Quality Control

RESUMO

Introdução: Acidentes com animais peçonhentos são classificados como doenças tropicais negligenciadas e são atualmente a mais frequente causa de intoxicação em humanos no Brasil. O único tratamento disponível é a rápida administração de antivenenos específicos e de qualidade garantida. Para assegurar a eficácia e a segurança desses produtos, são realizados ensaios de determinação da potência *in vivo* para veneno e antiveneno, desde as etapas de produção até sua liberação final. Apesar dos diversos estudos sobre métodos alternativos ao ensaio murino, nenhum método foi efetivamente validado. **Objetivo:** Compilar os métodos alternativos desenvolvidos para os antivenenos botrópicos, avaliando sua disponibilidade, perspectivas e aplicações em laboratórios de produção e controle da qualidade. **Método:** Foi realizada uma busca nas bases PubMed, BVS e Scopus entre novembro de 2021 e junho de 2022. Foram identificados 89 trabalhos, dos quais 31 foram selecionados de acordo com os critérios de elegibilidade. **Resultados:** Nos métodos alternativos identificados, observamos a preferência de 42,80% dos estudos por metodologias que utilizem linhagens celulares como método alternativo aos ensaios



murinos, sendo que a maioria destes trabalhos 58,30% optou pela linhagem celular Vero. **Conclusões:** Pela diversidade das toxinas encontradas em cada gênero de serpentes, entende-se que é de extrema importância que o ensaio de potência dos antivenenos tenha como base a avaliação e a quantificação precisa da inibição da atividade biológica dos venenos. Ensaio de citotoxicidade são amplamente utilizados e têm acumulado evidências de sua adequação como importante ferramenta alternativa ao ensaio murino para o controle da qualidade de veneno e antiveneno antibotrópico.

PALAVRAS-CHAVE: Métodos Alternativos; Veneno Botrópico; Antiveneno Botrópico; Controle da Qualidade

INTRODUCTION

Medically important snakes responsible for human envenoming accidents are found in about 160 countries and there is a high incidence, which is a serious public health problem in Africa, the Middle East, Asia, Oceania, and Latin America¹. Approximately 2.7 million snakebite envenoming occur annually in the world, causing 81,000 to 138,000 deaths per year. Almost 7,400 men, women and children are bitten daily and, among those injured, about 380 die². According to the latest data available from the National System of Toxic-Pharmacological Information, accidents with venomous animals have surpassed drug intoxications and are now the leading cause of intoxication in humans, with 35.25% of cases reported in Brazil³. Snakebites envenoming can kill up to six times more people compared to other diseases existing in tropical developing countries⁴. Currently, snakebites remain neglected, even though the incidence and mortality rates are higher than those of other neglected tropical diseases. Although there are a number of factors contributing to the high incidence, it was only in 2018 that snakebite accidents were elevated to Category A in the list of Neglected Tropical Diseases². The occurrence of these snakebites predominantly in poor countries is combined with other public health problems such as endemic diseases caused by vectors, parasitic diseases, and tuberculosis⁴.

In the goals of the 2030 Agenda for Sustainable Development⁵, it is envisaged to encourage international efforts to improve the availability and accessibility of safe and effective antivenoms for all. In this sense, it is essential that the production and quality of antivenoms meet internationally accepted standards, through cooperation between academy, industry, public and private institutions for innovation, and modernization^{6,7,8}.

Antivenoms are classified as biological products because they are produced from immunoglobulins obtained from animals hyperimmunized with specific antigens of one or more snake species. Consequently, these products may present variations that make it essential to analyze each batch produced⁹. Regarding the genus *Bothrops*, each milliliter of antivenom must contain enough immunoglobulins to neutralize 5 mg of the venom. In Brazil, the *Instituto Butantan* (IB) in São Paulo, the *Instituto Vital Brazil* (IVB) in Niterói/Rio de Janeiro, the *Fundação Ezequiel Dias* (Funed) in Belo Horizonte (MG), and the *Centro de Produção e Pesquisa de Immunobiológicos* (CCPI) of Paraná are the public laboratories responsible for the production of antivenoms, among other immunobiologicals. The National Institute for Quality Control in Health of the Oswaldo Cruz Foundation (INCQS

- Fiocruz) in Rio de Janeiro is the national reference public laboratory responsible for controlling the quality of antivenoms and products subject to health surveillance^{10,11,12}.

In all stages of the venom production until the release of the antivenom batches, animals are used, both by the producers and by the official quality control: snakes for venom extraction, rodents for feeding these snakes, large animals such as horses for the production of immunoglobulins and mice for testing Lethal Dose 50% (LD₅₀) and Effective Dose 50% (ED₅₀). According to the World Health Organization (WHO), there is an undeniable need for change and the implementation of 3Rs methods aimed at animal welfare^{5,13}. Important issues related to animal experimentation also include: (i) the genetic variability presented by the species of experimental animals in relation to the human species; (ii) the determinations regarding ethics in animal experimentation; (iii) the development of new alternatives to replace the use of animals in toxicological tests; and (iv) drug safety. In this context, several *in vivo* methods should be replaced by *in vitro* or *in silico* models, and in the impossibility of excluding animal models, an attempt has been made to use a smaller number of animals or even to use the same animals for different tests (procedure not allowed for tests with venoms)^{8,13}.

Since 2001, the WHO has recommended the development of alternative methods to the LD₅₀ and ED₅₀ murine lethality model. There is consensus that *in vivo* murine assays cause suffering to animals, are costly, and have little or no correlation with envenoming and therapy in humans. Thus, the WHO Expert Committee on Biological Standardization considers that “efforts should be directed towards the development of alternative methods for the substitution of rodent assays for the determination of the potency of antivenoms”¹⁴.

For decades, there have been calls, both by the scientific community and by entities linked to animal welfare, for the development of alternative tests capable of replacing the official methods of LD₅₀ and ED₅₀. However, there are controversies between the advantage of animal testing and the impact of animal experimentation (death, pain, and suffering). Currently, there are alternative methods capable of reducing the number of animals or that use *in vitro* systems to replace the murine lethality trial to determine the potency of antivenoms. Unfortunately, these systems are not yet officially validated as a substitute for murine assays^{5,16}. However, it should be considered that *in vivo* or *in vitro* assays have inherent technical limitations. The protocols



of venom and venom/antivenom inoculation intraperitoneal in mice do not reproduce the natural situation. Accidents affect the lower limbs in 80% of cases, in addition to involving inoculation by intradermal and/or intramuscular route, while in mice, the test is performed intraperitoneal or intravenous¹⁷. Therefore, care must be taken to avoid simplistic extrapolations from these assays to the clinical situation. Furthermore, the physiological response of rodents to envenoming and therapy may differ significantly from that of humans. These limitations make the murine model reproduce envenoming and the treatment in humans less than ideal¹⁸. However, the LD₅₀ and ED₅₀ assays remain the official methods for potency determination and are still widely used because they are the only assays described in the Brazilian Pharmacopoeia^{11,15,16,19} and several clinical studies have shown that the ED₅₀ is very useful, but not infallible, in predicting the effectiveness of antivenoms in clinical practice.

The development of alternative methods to the use of animals in the evaluation of the potency of antivenoms should be encouraged, making it necessary that the potential alternatives be carefully evaluated to guarantee the validity of the results. Research should be carried out to develop both the refinement of the protocols of the *in vivo* assays and the *in vitro* alternatives. The results of any modification of the protocols or alternative protocols must be rigorously compared with the results of the official methods, to guarantee the statistical validity of the proposed methods¹⁶.

Although there have not been coordinated and standardized initiatives to validate the *in vitro* tests to replace the use of animals in *Bothrops* antivenoms, the scientific literature presents several independent studies, presenting evaluations of different methods. Knowing this evidence can bring a greater understanding of its suitability, effectiveness, and limitations, pointing out ways for future validation studies. In this context, this review aimed to compile alternative methods developed for *Bothrops* antivenoms, evaluating the availability of methods, their perspectives, and applications in the quality control for *Bothrops* venom and antivenom.

METHOD

A survey was carried out between November 2021 and June 2022 in the following databases: PubMed (Medline), Virtual Health Library (VHL), and Scopus. During the electronic search, the following free terms were used: “*Bothrops*”, “*Bothrops jararaca*”, “antivenom”, “*in vitro*”, “cell based”, “potency”, “potency evaluation” e “neutralizing potency”, generating the following search keys:

- PubMed: (*Bothrops jararaca* or *Bothrops* or jararaca) [tiab] AND (*in vitro* or cell based) [tiab] AND (potency or neutralizing potency) [tiab];
- BVS: (*Bothrops jararaca* or *Bothrops* or jararaca) AND (*in vitro* or cell based) AND (potency or neutralizing potency);
- Scopus: (*Bothrops* AND “*in vitro*” AND neutralizing potency).

Eligibility criteria

Eligibility criteria for inclusion of studies included articles in English, Spanish, or Portuguese, according to the modified IOP criteria, where: I (intervention) = *in vitro* tests; O (outcome) = determination of *Bothrops* antivenom potency, P (population) = cell culture, plasma, eggs;

The following exclusion criteria were applied to the studies: studies on the effect of the venom on other organisms, studies in which the *in vitro* trial consisted only of pre-incubation for the *in vivo* trial, studies in which the purpose was to evaluate molecules, mediators of inflammation, the effect of a venom component and therapeutic targets, refinement studies of the *in vivo* method, in addition to off-topic articles.

Data selection and extraction

All article titles and abstracts were evaluated and selected according to eligibility criteria. Duplicates were excluded using the Zotero® reference manager that compares titles, the International Standard Book Number (ISBN), and the Digital Object Identifier (DOI). Then, the articles that were not excluded during the evaluation of the abstracts were read and judged according to the eligibility criteria, type of methodology performed, main results obtained, and perspectives for implementation. For data extraction, scientific and technical information items were tabulated and analyzed in Microsoft Office Excel 2013. The extracted data included the year of study and publication, author, biological matrices, countries of origin of snakes, methodology used, study purpose, and study outcomes.

RESULTS

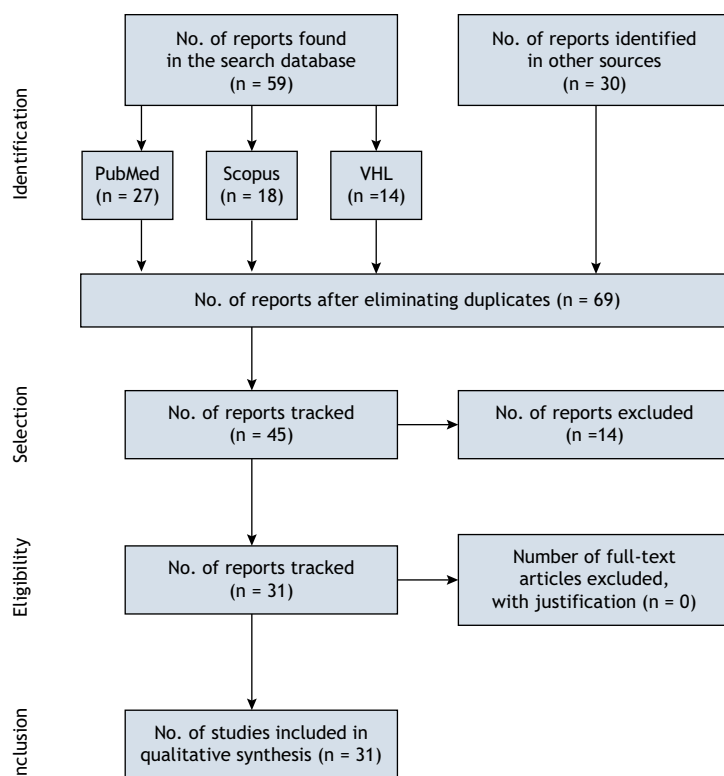
Database search

The electronic search found 89 scientific papers. A patent record was found in another search source and manually included in the selected articles. After the exclusion of duplicates, a total of 69 studies were evaluated for eligibility by applying the exclusion criteria. Of these, 24 did not meet the criteria previously defined and 45 studies were later analyzed. After the complete reading, 31 studies were selected. Therefore, 58 were excluded from the study because they did not meet the eligibility criteria (Figure 1).

Analysis of selected articles

In the Chart, the characteristics of the included studies are described, such as: author, year of publication, objective of the study, and the main findings.

Analyzing the 31 selected articles, three of them are reviews^{7,9,49}, so 28 effectively used biological matrices in tests. We observed a preference of 42.80% of the studies for methodologies that use cell lines as an alternative method to the murine assays (12 of 28 articles)^{13,21,22,23,24,25,26,27,28,29,30,31}, 25.00% used plasma^{32,33,34,35,36,37,38},



Source: Elaborated by the authors, 2022.
No.: number; VHL: Virtual Health Library.

Figure 1. Flowchart of the selection process and selection of articles using the Prisma model²⁰.

14.28% used venom^{42,43,44,49}, 10.70% used gelatin^{39,40,41}, 7.10% used enzymes^{45,46}, while a single study (3.50%) used antibodies⁴⁷ and another used blood on filter paper⁴⁸.

Another approach is the assays found that measure blood clotting^{32,33,34,35,38}. Of these, four studies used human plasma^{32,33,35,38}, one study used avian plasma³⁴, and a human plasma study performed a trial with bovine fibrinogen³³ in parallel. Another three assays measured the diameter of the hemolytic halo in the degradation of agarose gel^{39,40,41}. We found assays comparing two methods^{24,25,48} and even three methods^{34,45,47}.

However, new venom techniques are being investigated seeking to elucidate the complexity in the composition of venoms, in the identification of interspecies and intra-species variations observed from the geographic origin and developmental stage of the snakes^{42,43,44}. The study of crude venom proteins and peptides is called proteomics and allied to this technique are databases and algorithms facilitating the mapping of the composition of each venom⁴⁹.

It should be noted that most authors performed cytotoxicity studies, through which they evaluated the metabolic function or membrane integrity in viable cells previously exposed to *Bothrops* venom, while other studies addressed the number of living cells after exposure. Were used: muscle cell lines (C2C12), mimicking the action of the venom on the muscles; renal cell

lines (Vero) that can mimic the toxic effects of *Bothrops* venoms; murine macrophage cells obtained from the peritoneal cavity (RAW 264.7) known to be involved in inflammatory and necrosis processes, mouse endothelial cells that form the inner lining epithelium of veins and heart (tEND), tumor cells (MGS0-3), in addition to cells already standardized in viability studies such as HeLa.

The methodologies used were: I) MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) reduction method in which the dye is reduced to formazan only in living cells; II) Trypan blue method that evaluates the number of viable cells; III) Measurement of the release of lactate dehydrogenase (LDH), an enzyme that is released when damage to the plasma membrane occurs; IV) Method of converting resazurin to resorufin by living cells; V) Cell quantification technique with Coomassie Brilliant Blue (CBB R-250) dye that stains cellular proteins and lipids.

Among the 28 studies, 18 compared *in vitro* methods with *in vivo* method^{13,21,22,23,24,26,29,30,31,33,34,35,37,39,40,41,47,48} (Figure 2). Most studies showed a good correlation between the *in vitro* and *in vivo* method, however, Maria et al.⁴¹ found no correlation for Brazilian *Bothrops* antivenoms, despite the high correlation between Elisa titers (enzyme immunoassay) and neutralization of lethality when using the toxic fraction to coat the plates. According to Oliveira et al.²³, there is a significant correlation



Chart. Articles included in the review and their characteristics according to author, year of publication, study objective, and main findings.

Author	Year	Objective of the studyObjetivo do estudo	Main findings
Williams et al. ⁷	2019	Review of the challenges and strategies to be adopted for the prevention, control, and reduction of accidents with snakes.	They compiled the main global actions to be coordinated by the WHO based on four pillars: I) ensure safe and effective treatment, accessible to all; II) empower and engage communities; III) strengthen health systems; IV) fundraising for the implementation of the roadmap.
Nundes ¹³	2017	Pre validation of an <i>in vitro</i> potency assay for <i>Bothrops</i> venom and antivenom.	The proposed method demonstrated the ability of <i>Bothrops</i> venom to cause a cytotoxicity effect on Vero cells and proved reliability in intra-assay and inter-assay precision. The results certify the methodology to be followed in the validation process (interlaboratory reproducibility).
Lomonte et al. ²¹	1994	Endothelial cell cytotoxicity assay based on LDH release for <i>Bothrops asper</i> venom.	The BaH-1 toxin, despite its potent <i>in vivo</i> hemorrhagic action, did not induce significant cytotoxicity, however, myotoxin II was cytotoxicity under identical trial conditions.
Lomonte et al. ²²	1999	Cytotoxicity assay in C2C12 myotubes based on LDH release for <i>Bothrops asper</i> venom.	Skeletal muscle myotubes, obtained after differentiation of C2C12 myoblasts were significantly more susceptible to the cytotoxicity action of myotoxins than endothelial cells.
Oliveira et al. ²³	2002	Correlate the 50% cytotoxicity dose (CT ₅₀) of eight venoms in Vero cells with the 50% lethal dose (LD ₅₀) and correlate the CT ₅₀ with MPD (minimum proteolytic dose).	<i>B. neuwiedii</i> mattogrossensis and <i>B. leucurus</i> were the most cytotoxicity, while <i>B. atrox</i> and <i>Bothrops</i> sp were the least cytotoxicity venoms. A significant correlation was obtained between CT ₅₀ and LD ₅₀ by intravenous injection, except for <i>B. n. mattogrossensis</i> . A positive correlation was also found between CT ₅₀ and LD ₅₀ and between CT ₅₀ and MPD.
Zobiole et al. ²⁴	2015	Perform cytotoxicity (MTT) and genotoxicity assay in Vero cells for <i>Bothrops moojeni</i> venom.	The venom showed cell concentration-dependent cytotoxicity with a value of CC ₅₀ obtained from 4.09 ± 0.04 µg/mL and was also genotoxic to Vero cells at 4 µg/mL.
Menezes et al. ²⁵	2016	Perform MTT and LDH cytotoxicity assay for <i>Bothropoides insularis</i> venom.	The venom causes cell death and proliferation in macrophages, depending on the concentration and exposure time. Cytotoxicity was also confirmed by the measurement of LDH, both in clinical and experimental samples.
Lopes-Souza et al. ²⁶	2019	Development of an <i>in vitro</i> method to determine the potency of <i>Bothrops</i> antivenom.	The venom concentration capable of reducing 50% of cell viability was 11.79 µg/mL. There was a decrease in cell viability dependent on venom concentration.
Rodrigues et al. ²⁷	2018	Analyze the composition of the venom of <i>B. brazili</i> . The enzymatic activities were analyzed <i>in vitro</i> .	A total of 189 proteins were identified. <i>B. brazili</i> venom showed high activity of SVSP, but low activity of PLA 2, when compared to other <i>Bothrops</i> venom. In addition, <i>B. brazili</i> venom reduced viability in VERO cells.
Rodrigues et al. ²⁸	2020	Analyze the components of the <i>B. brazili</i> venom. Venom cytotoxicity was tested on Vero cells.	About 37 <i>B. brazili</i> proteins were identified. This study provides an overview of the venom composition of <i>B. brazili</i> and demonstrates the effectiveness of Peruvian and Brazilian antivenoms in the treatment of accidents with this species.
Mora-Obando et al. ²⁹	2014	Study the synergism between two phospholipase enzymes from the <i>B. asper</i> venom in C2C12 cells.	At both stages of cell differentiation, the combination of the myotoxins induced significantly greater cytotoxicity compared to the effect of either toxin alone. The observed effect was clearly synergistic and not just additive.
Lopes-Souza et al. ³⁰	2015	Cytotoxicity assay of five venoms on MGSO-3 cells. Proteolytic activity was tested <i>in vitro</i> and correlated with previously published <i>in vivo</i> data.	The study confirmed that <i>Bothrops</i> venom toxins are distributed differently in different species. Knowledge of such differences can be of great relevance to understanding the effects of snakebite envenoming and antivenom production. The results obtained indicate that a battery of <i>in vitro</i> methods can be used to determine the main toxic activities of <i>Bothrops</i> .
Guerra-Duarte et al. ³¹	2015	Characterize 11 venoms from Peruvian snakes of the genus <i>Bothrops</i> through a cytotoxicity trial with three cell lines (MGSO3, Vero and HeLa).	The venoms were able to reduce cell viability in all tested strains. Each venom showed a specific pattern of toxicity, so they were divided into four groups according to their composition. The venoms in group one were the least cytotoxicity and had the lowest amount of LAAO, while those in group four were the most toxic and had the highest amounts of LAAO. There was a correlation between these values, while for other enzymatic activities no correlation was found.
Demple et al. ³²	1990	Analyze and compare a picture of human envenoming in parallel with an <i>in vitro</i> coagulation assay.	<i>B. neuwiedii</i> causes concentration-dependent clotting of fibrinogen. The <i>in vitro</i> and <i>in vivo</i> results showed reduction in Factors II, V, and X in human plasma after snakebite from <i>B. neuwiedii</i> . Administration of antivenom accelerates the elimination of venom proteases from the patient's bloodstream.
Laing et al. ³³	1992	Compare the efficacy of three batches of <i>Bothrops</i> antivenoms <i>in vivo</i> and <i>in vitro</i> coagulation assays.	Antivenoms were equally effective in neutralizing <i>in vitro</i> plasma clotting activity, <i>in vitro</i> fibrinogen clotting activity, and <i>in vivo</i> and necrotizing activity.

Continue



Continuation

Oguiura et al. ³⁴	2014	Determine the minimal coagulant dose, procoagulant activity of the venom, and the effective dose of the antivenom using the ROTEM assay.	The concentrations of <i>Bothrops</i> venoms that induce the coagulation of chicken citrate plasma in 60 seconds are significantly higher compared to those presented when measured in human plasma.
Chacón et al. ³⁵	2015	Compare the neutralization of coagulant activity in citrate human plasma to the neutralization of murine lethality assay.	There was a significant correlation between the neutralization of lethality and the neutralization of clotting activity <i>in vitro</i> for the <i>B. asper</i> venom.
Martins et al. ³⁶	2009	Evaluate the hemolytic activity <i>in vitro</i> of the venom and phospholipases A2 of <i>B. lanceolatus</i> .	Both <i>B. lanceolatus</i> venom and isolated phospholipases A2 can cause indirect hemolysis of erythrocytes in sheep, mice, cows, and horses. Horses are the most susceptible. Venom incubation at different temperatures indicated that the component of the venom responsible for hemolysis is thermolabile.
Rial et al. ³⁷	2006	Determine the potency of <i>B. alternatus</i> antivenom in blood samples from horses hyperimmunized with antivenoms and Fab'2 fractions.	A significant correlation was found between Elisa values and <i>in vivo</i> test. The main advantage of the method was being able to analyze the neutralization capacity of hyperimmune plasma and antivenom, regardless of whether it is total IgG or Fab2 fraction.
Estevão-Costa et al. ³⁸	2016	Evaluate the effectiveness of the <i>Bothrops</i> antivenom produced at <i>Instituto Nacional de Salud</i> (INS, Peru) and at <i>Fundação Ezequiel Dias</i> (Funed, Brazil) to neutralize the main toxic activities of five <i>Bothrops</i> venoms	The electrophoretic patterns of the proteins of these venoms showed significant differences in the composition, number, and intensity of bands. Both antivenoms are equally effective in neutralizing <i>Bothrops</i> venom and cross-reacted to venoms not included in the formulation as <i>B. atrox</i> venom.
Gutiérrez et al. ³⁹	1988	Perform pre-incubated venom/antivenom assay and inoculate into agarose gel containing sheep erythrocytes and egg yolk to measure hemolytic halo diameter and correlation with <i>in vivo</i> assay.	A linear dose-response was observed. Several batches of antivenom neutralized hemolytic activity, indicating that the neutralization occurs due to a specific immune reaction. It also showed a significant correlation in 15 samples of antivenoms capable of neutralizing indirect hemolysis and neutralizing lethality.
Heneine et al. ⁴⁰	1988	Development of Elisa immunoassay for determination of <i>Bothrops</i> antivenom potency and correlation with <i>in vivo</i> assay.	Two fractions of the venom with hemorrhagic activity and two fractions with phospholipase activity showed a correlation with the antivenom. The results indicate that the use of less complex antigens can be applied in development of alternative methods for potency determination for <i>Bothrops</i> antivenom.
Maria et al. ⁴¹	1988	Correlate the <i>in vivo</i> assay with the neutralization of the hemolytic activity of phospholipases A2.	No correlation between lethality neutralization and Elisa titers against crude venom was observed for Brazilian <i>Bothrops</i> antivenoms. However, a high correlation was found between Elisa titers and neutralization of lethality when using the toxic fraction of the venom.
Sanz et al. ⁴²	2020	Perform venomomics and antivenomics of <i>B. brazili</i> .	In addition to contributing to the characterization of the <i>B. brazili</i> venom, this study demonstrated a specific immunoreaction of the Brazilian <i>Bothrops</i> antivenom with all the <i>B. brazili</i> toxins.
Gutierrez et al. ⁴³	2009	Antivenomic review.	Proteomics characterizes venoms in relation to the toxicological profiles of each species. This can help a lot in designing customized assay for each venom. It can contribute to the introduction of different <i>in vitro</i> tests and the reduction of animal use associated with <i>in vivo</i> tests. Since proteomics technologies are not available in many countries, the organization of academic and public health partnerships should be promoted, involving laboratories with technology and resources, but at the same time empowering low-income countries in Asia, Africa, and Latin America.
Mora-Obando et al. ⁴⁴	2021	Application of antivenom to compare the specific and paraspecific immunoreactivity of six <i>Bothrops</i> antivenoms against three Colombian venoms.	The results agree with previous studies in revealing the ability of <i>Bothrops</i> antivenoms to neutralize each other in preclinical tests, Central and South American <i>Bothrops</i> venoms, and highlight quantitative differences in their ED ₅₀ . The combination of antivenom and <i>in vivo</i> neutralization assays provides relevant information to delineate the species and geographic range of clinical applicability of an antivenom.
Mendoza et al. ⁴⁵	2008	Analyze the inhibition of phospholipases A2, LAAO, and hyaluronidase activities as an indicator for potency for antivenoms <i>in vitro</i> .	Exposure of the antivenom to temperatures above 25°C showed that the antivenom in the liquid state underwent changes. It was concluded that a dose of the three enzymes studied, but mainly of hyaluronidase, could serve as an <i>in vitro</i> indicator for the potency for antivenom produced by the INS.
Avila et al. ⁴⁶	2010	Develop an <i>in vitro</i> assay using the LAAO enzyme from the <i>B. jararaca</i> venom as antigen for Elisa and determine the potency for <i>Bothrops</i> antivenom.	Patent: PI 1004449-3 A2 ⁵¹ .

Continue



Continuation

Rafael et al. ⁴⁷	2008	Perform Elisa assay with mono and polyclonal anti-jararagin antibodies capable of detecting metalloproteases in a pool <i>B. jararacussu</i> venom.	A significant correlation was obtained between the hemorrhagic activity samples and their reactivity with the MAJar 3, demonstrating the potential of the Elisa method as an alternative to the <i>in vivo</i> hemorrhagic tests. It suggests that the immunoassay can be a reliable tool in determining the bleeding levels of metalloproteases in snakebite victims.
Maria et al. ⁴⁸	2001	Determine the potency for 16 antivenoms in blood samples on filter paper by the Elisa method and its correlation with the <i>in vivo</i> assay.	The results indicate that the Elisa assay with blood samples on filter paper can be an alternative method to access the potency for antivenoms during equine immunization. This methodology is already commonly used in serological tests, as it greatly facilitates the collection, transport, and storage of samples.
Tan ⁴⁹	2022	Antivenomic review.	It characterizes the different venomomics methodologies: I) Proteomics - the study of crude venom proteins and peptides; II) Transcriptomics - the study of RNA or DNA extracted from the venom-secreting gland; and III) Genomics - the study of the genome sequenced from the extraction of any snake tissue. It also discusses the challenges to the advancement of these techniques due to the constant need to improve the methodology, update the databases, and include lesser-known species.

Source: Elaborated by the authors, 2022.

WHO: World Health Organization; LDH: lactate dehydrogenase; B.: *Bothrops*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LAAO: L-amino acid oxidase; Elisa: enzyme immunoassay; CT₅₀: cytotoxicity dose 50%; LD₅₀: lethal dose 50%; MPD: minimum proteolytic dose; SVSP: serine proteases; ROTEM: thromboelastometry; sec: seconds; ED₅₀: effective dose 50%; INS: *Instituto Nacional de Salud*.

between the cytotoxicity dose (CT₅₀) and the median lethal dose (LD₅₀) by intravenous injection, except for venoms of *Bothrops neuwieddi mattogrossensis* and *Lachesis muta*. Analyzing the correlation between CT₅₀ and LD₅₀ intraperitoneal or between CT₅₀ and the minimum proteolytic dose (MPD) a positive correlation was also found, however two species did not adjust in both cases: *Bothrops atrox* and *Bothrops* sp. These results are corroborated by studies^{23,35,39} that reinforce the specificity of venoms and the need for differentiated assays.

Mora-Obando et al.²⁹ observed that the toxic effect of two venom myotoxins caused a significant increase in the cytotoxicity effect in myoblasts, myotubes, and in mice compared to the effect of either toxin alone. This work advocates that not only the action of purified toxins be analyzed, but also the synergism that occurs between the different toxins of the venom, potentiating its toxic effect.

Of the 31 studies presented in this study, 17 exclusively used the raw venom, the venom pool, or the venom fractions of a single snake species^{13,21,22,24,25,26,32,33,35,36,39,40,41,45,46,47,48}, 12 were carried out with snakes of the species *Bothrops jararaca*^{13,23,26,30,33,34,38,40,41,42,46,48}, six works, with snakes of the species *Bothrops asper*^{21,22,29,35,39,44}. Some authors used more than one species in the same study. Only 11 of the 31 studies carried out studies with antivenoms^{13,21,23,24,25,26,27,28,38,42,44}. Three studies were carried out with antibodies from hyperimmunized horses^{36,37,48} (Figure 3).

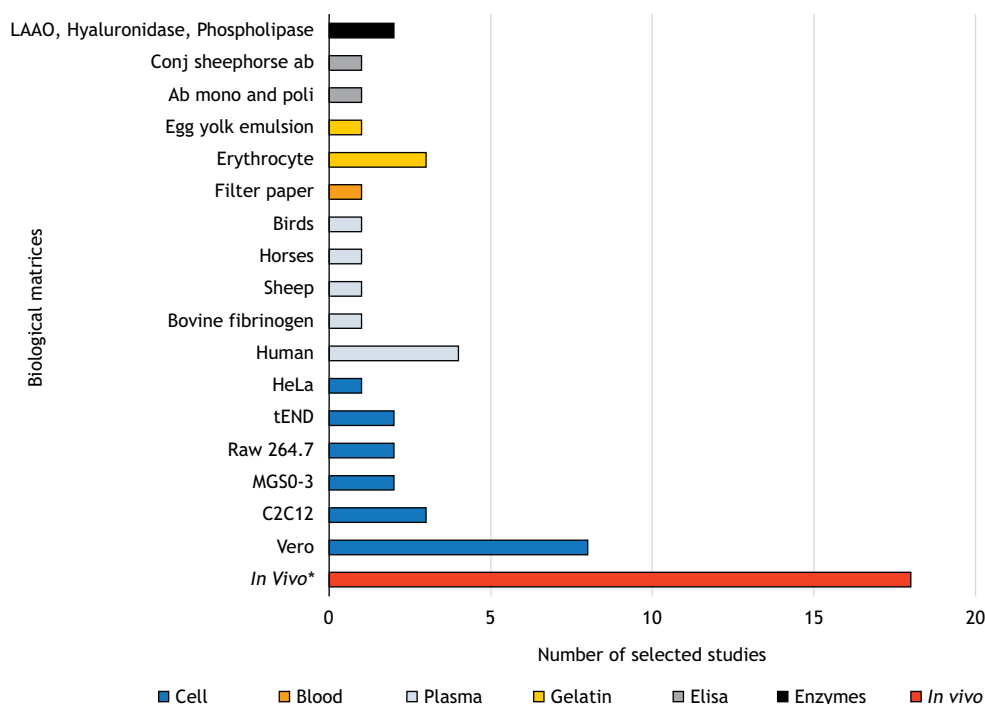
We can observe that, of the 31 selected studies, 15 used snake venom from Brazil^{13,23,24,25,26,28,33,34,38,40,41,42,46,47,48} and one study used *Bothrops jararaca* venom as control³¹, six studies used venom from Costa Rican snakes^{21,22,35,39,44,29}, seven were carried out with venom from Peruvian snakes^{23,27,28,31,38,44,45}. Three studies were carried out with venom from snakes from different Latin American countries: Argentina, Peru, Brazil, and Bolivia²³, Peru and Bolivia³⁸, and Colombia⁴⁴.

Of the 31 related studies, 15 are on the development of alternative methods^{21,22,24,25,27,28,29,31,32,33,36,38,42,43,44}, 12 selected studies aim to replace animal testing in production/industry^{23,26,30,34,35,37,39,40,41,45,46,47}. Most of these studies suggested partial replacement only during the first stages of antivenom production: in horse immunization and in plasma fractionation processes. They also suggested that the LD₅₀ trial remain in the last stages of antivenom production and for the quality control of the final product³⁵, and they also recommended testing in mice to release antivenoms lots⁴¹. Only two studies aimed to substitute for quality control^{13,34} (Figure 4).

Alternative methods for antivenom potency found in this selection evaluated specific antibodies against venom toxins or toxins that showed a correlation between *Bothrops* accidents, *in vivo* neutralizing activity, and *in vitro* results. Most of the *in vitro* tests were performed in triplicate intra-assays, except for: Maria et al.⁴¹, Guerra-Duarte et al.³¹ and Rodrigues et al.²⁸, who only performed intra-assay duplicates. Another exception was the use of the European Pharmacopoeia formula⁵⁰ by Henene⁴⁰ while the standard *in vivo* trial uses the Brazilian Pharmacopoeia¹¹. A wide variety of negative controls used in the studies were observed: saline, phosphate-buffered saline (PBS), water, non-immune serum, and antivenoms.

DISCUSSION

The first data compiled by the WHO on worldwide mortality due to snakebite envenoming were published in 1954, with an estimated 30,000 to 40,000 deaths per year worldwide. From that time to the present day, it is known that the number of cases is much higher, because many data are underreported⁵¹. Only 55 years after the first publication⁵¹ was due importance given to snakebite envenoming, and these were included by the WHO in the list of Neglected Tropical Diseases. In 2010, the WHO defined antivenoms as essential medicines



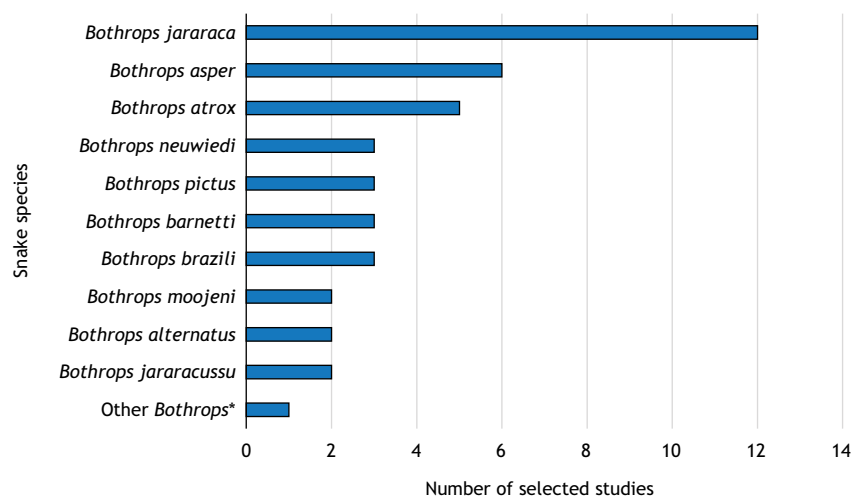
Source: Elaborated by the authors, 2022.

**in vivo*: corresponds to the 18 selected studies that compare *in vitro* assays with *in vivo* assays.

Obs: Some studies used more than one cell line.

LAAO: L-amino acid oxidase; Conj sheephorse ab: antibody conjugated sheephorse; Mono and poly Ab: monoclonal and polyclonal antibody.

Figure 2. Biological matrices used in the selected articles.



Source: Elaborated by the authors, 2022.

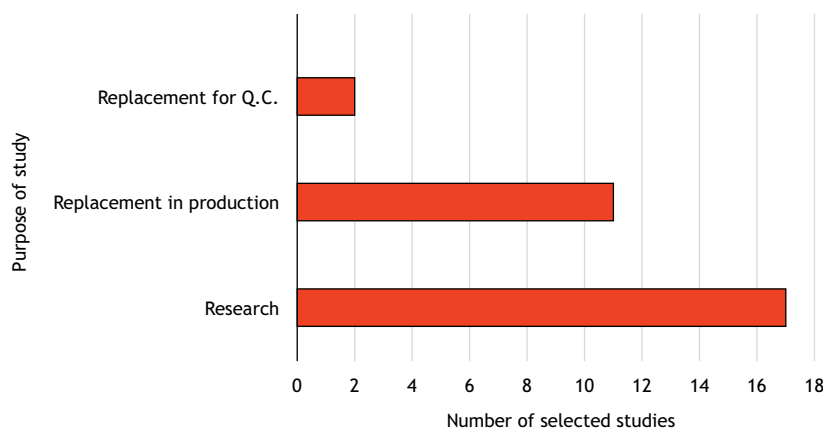
Other *Bothrops**: *Bothropoides insularis*, *Bothriopsis bilineata smaragdina*, *B. nummifer*, *B. godmani*, *B. lanceolatus*, *B. pubescens*, *B. leucurus*, *B. rhombeatus*, *B. ayerbeii*, *B. andianus*, *B. castelnaudi*, *B. chloromelas*, *B. microphthalmus*, *B. oligolepis*, *B. peruviana*, *B. taeniata*. The venoms of these species were used only once.

Figure 3. *Bothrops* snake species in the selected studies.

and published guidelines for their respective production. From this publication, the first guidelines emerged on the need to improve the quality, safety, and regulation of antivenoms - the only available treatment for snakebite envenoming. In it, there is an emphasis on the efforts made by various groups

to implement the concept of the 3Rs in the development and quality control of antivenoms and, in 2017, there was a review of this publication^{1,2,6,16}.

The first time that the WHO mentioned the importance of alternative methods for replacing rodents in assays to determine



Source: Elaborated by the authors, 2022.
Q.C.: Quality Control

Figure 4. Purpose of selected studies.

the potency of antivenoms was in 2001¹⁴. Although the desired objective is the replacement of animals, while validations are taking place, efforts must be made to ensure the refinement of the technique and the reduction of the number of animals in these tests. Also in 2017, the European Medicines Agency (EMA)⁵² published a Guide to regulatory acceptance of assays with 3Rs Approaches to in-process control for human and veterinary drugs. This publication aimed to guide and encourage the development of tests based on the replacement, reduction, and refinement of animal experimentation. This document lists the criteria for regulatory acceptance of adaptations in tests already existing in official compendia or new tests provided that one of the 3Rs is applied. Even so, it is necessary to certify the methodology through standardized protocols with scientifically accepted parameters, to demonstrate the precision and relevance of the method, that is, the method must be effective for the biological purpose that the test proposes to prove. Then, the methodology must be ratified as reliable, robust and reproducible through interlaboratory studies and, finally, prove that the method is useful for the purpose created, being as good or better than the current methodology in the prediction of the tested effects. Despite the great diversity of articles found, none of these methods was submitted to an interlaboratory study or validation against the current official methodology (murine lethal model) to determine the potency of *Bothrops* antivenom. The selected studies were only up to the development, standardization, or pre-validation stages. Currently, the challenge is to carry out a proper validation study to achieve regulatory acceptance.

Since then, the WHO has shown great concern about this public health problem, so much so that it has convened experts in the area to develop strategies to be implemented in the coming years until 2030. Among all the definitions is the 50% reduction in deaths and disabling sequelae through the availability of safe and effective antivenoms that are guaranteed by quality control^{2,5}.

In this review, it was possible to identify that there are published studies on alternative methods for antivenoms with

promising results: presenting a significant correlation between the biological activity of the studied venom and the neutralization of the *in vitro* activity^{13,23,27,30,31,35,37,39,40,41,47,48}. However, due to the specificity of the venoms of each genus of snakes and their medical importance related to their geographical location, studies were carried out with several methodologies, using different controls and heterogeneous exposure times. This diversity makes it possible to identify key events and thus suggest an integrated testing strategy. In addition to publications, there are many working groups in different research and teaching institutions, expanding the possibilities of partnerships to fill the gaps in this great challenge.

Gutiérrez et al.⁸ brought an important review on the complexity related to *in vitro* tests regarding the purpose and pre-clinical efficacy of antivenoms. The pathophysiology of *Bothrops* envenoming occurs through the action of isolated toxins or synergistically in which their toxic effect is potentiated. There are also two routes of action of the toxins: at the bite site (firm swelling, pain, bleeding, necrosis) and/or systemic (hemorrhage, hypotension, renal failure). Furthermore, after inoculation of the venom, each organism reacts to the introduction of the venom with a cascade of inflammatory reactions. As for in-process quality control assays, *in vitro* assays are recommended both for the stages of antibody production in horses and for quantitative analysis of neutralizing antibodies during plasma fractionation.

Discoveries of immunity mechanisms for sera date back to 1890, when Behring and Kitasato published the first study on the development of immunity to diphtheria and tetanus in animals. Just two years after this publication, the first human trial was performed with antidiphtheria serum^{53,54,55}. In practice, the use of tetanus serum was consolidated in 1914 due to the need to administer it to soldiers wounded in the First World War⁵⁶. The method of inhibiting the binding between toxin and antitoxin (ToBI) is a successful example of an alternative method validated and included in the European Pharmacopoeia both for the stages of production of tetanus vaccines and for quality



control^{57,58}. INCQS and IB validated the potency test for anti-rabies serum (RFFIT)^{11,59}.

Hyperimmune sera are biological products that, as a result, vary with each batch produced. In the last decades, several alternative methods to the use of animals have been developed based on the concepts of the 3Rs. As for antivenoms, although several methods have been developed, none was able to complete the validation process.

The studies listed below can be divided into two areas: (i) the research area, with a clinical view and drug development for other purposes; and (ii) studies related to a regulatory view, such as pre-clinical studies for the registration of new products for in-process quality control in the stages of antivenom production and batch release.

For quality control, a patent application number PI 1004449-3 A2⁴⁶ was found, referring to a kit for determination of *in vitro* potency. This kit consists of plates seeded with the L-amino acid oxidase (LAAO) enzyme from the *B. jararaca* snake venom, responsible for toxic effects of the venom such as: hemorrhage, inflammation, and edema. This enzyme was used as an antigen to sensitize Elisa plaques and determine the neutralizing potency of *Bothrops* antivenoms.

Seels developed a trial in embryonated eggs with six days of incubation, that is, in the initial period of embryonic development, which is known to be a period when embryos do not feel pain. This test is widely studied for other outcomes as an alternative method for eye irritation and is accepted by the Animal Ethics Committee (CEUA) if performed up to the 9th day of incubation. The trial is performed by inoculating the mixture of venom and antivenoms into the vascularized region of the yolk sac. The advantage of this method is the observation of the hemorrhagic effect on discs containing venom and the neutralization of the hemorrhagic effect by the disc containing antivenom. In addition to visualizing the toxic effects of the venom, this method provides recording for further analysis and comparison. As embryonated eggs are in the period before neurological development, neurotoxic venoms cannot be tested^{15,60,61}.

Chacon et al.³⁵, in agreement with Pornmuttakun and Ratana-banangkoon⁶² and Oguiura et al.³⁴, described how the neutralization of coagulant effects is correlated with the potency of antivenoms. These studies take into account that the extravasation induced by metalloproteases seems to be the main factor behind the lethal effect of the *Bothrops asper* venom, therefore antibodies against hemorrhagic metalloproteases would also be effective against this procoagulant enzyme. Although they observed a correlation between neutralization of *in vitro* coagulant effects and lethal activity for the *B. asper* venom, this effect may not happen for all venoms. Therefore, tests must be carried out for each venom/antivenom respecting its intrinsic specificity.

Some techniques have already been developed by laboratories working with venoms and antivenoms to replace animals in quality control in production processes, such as immunochemical

methods (antivenomics and Elisa) to screen for antivenoms and evaluate antibodies in hyperimmunized horses, in addition to *in vitro* methods that evaluate the toxic activities of venoms that show good correlation with methods *in vivo* (enzymatic activities, cell culture, coagulation test, receptor binding, and neuromuscular preparation)^{4,63,64,65}. Despite the variety of pathophysiological activities present in the venoms of *Bothrops* and *Lachesis*, it has been shown that there is a correlation, for some venoms, between CT₅₀ and LD₅₀, and CT₅₀ and MPD^{24,66}.

Chacon et al.³⁵ correlated the *in vivo* trial with the *in vitro* trial of human plasma clotting activity. This innovation, when put into practice, will also allow a reduction in the number of animals for quality control in the production stages. Techniques for reduction should be encouraged and validated as we seek replacement by *in vitro* methods.

Guerra-Duarte et al.³¹ tested cell viability using the Alamar Blue® reagent in three cell lines (Vero, MGS03, HeLa) after exposure to serial dosages of venoms. The results showed that each venom presented a specific pattern of toxicity with dose-response curves presenting a similar format for all tested strains. Since the 1990s, the first alternative studies for venom in cell culture have been observed but, from the 2000s, this approach has reappeared, proving to be a promising methodology. Some factors may contribute to this approach, both because of their low cost and because they are methodologies that are already widely used in quality control. As an example, we have the fact that vaccines against dengue, HPV, yellow fever, and MMR are analyzed in cells¹³.

Lopes-Souza et al.³⁰ performed cytotoxicity assay in MGS03 cells with the venom of the five main Brazilian *Bothrops* species. The proteolytic activity of venoms was tested *in vitro* and correlated with previously published *in vivo* data⁶⁷; as Nundes¹³ correlated the cytotoxicity study in Vero cells with the *in vivo* study by Araújo et al.^{66,68}. Rodrigues et al.²⁷ compared the results obtained with previous studies that used the same methodology^{27,31}. The use of pre-existing data making a retrospective analysis of the *in vivo* data seems to be a strong tendency to avoid carrying out further animal tests.

Statistical methods were used to determine test results, such as: linear regression analysis of dose-response curve^{13,23,24,26,30,31,34,47} or Probit transformation^{35,37,41}.

Most authors performed the statistical analysis by determining the accuracies, intra and inter-assays, by calculating the variance (geometric variation coefficient), the agreement between the results of the *in vivo* and *in vitro* methods, and the sensitivity of the proposed method. Some studies compared treated groups with positive and negative controls by analysis of variance (ANOVA)^{24,26,30,34,35}, Newman-Keuls comparison test^{24,34}, Student's t test^{26,35}, Bonferroni's test²⁶, Paired t test³³, Turkey test³⁵, and Pearson's correlation coefficient^{37,47}.

To calculate the accuracies, the following programs were used: GraphPad Prism^{®13,24,26,30,31,34}, SigmaStat^{®40} for regression and statistical analysis, and CombiStats^{®13}.



Based on all the results found, intra-assay accuracies were evaluated, that is, assessment of the agreement between the results of the same analyst, on the same day and in one assay. Inter-assay precision was also calculated by evaluating the results performed on different days. For biological assays a coefficient of variation of up to 20% as in Elisa is an acceptable value⁶⁹. Therefore, it is essential to carry out a next step to assess interlaboratory reproducibility.

The most used methods to compare two measures of the same variable are: the Bland-Altman plot, the Deming regression, and the Lin concordance correlation coefficient (CCC). This is especially important if the objective is to introduce a new measurement method that has some advantages (cost reduction or biosafety) over an existing measurement technique (the “gold standard”).

A modern approach to trial comparison is the understanding that the surrogate method should not be required to have a significant correlation with the reference method, since both the *in vivo* trial and the *in vitro* assays have inherent variability. The new test must be in agreement with results with *in vivo* potency and must be able to discriminate between potent and subpotent (true-negative) batches. That is why some authors refer to “concordance” instead of “correlation”^{70,71}.

The three essential pillars to ensure the safety and efficacy of a product are: pre-clinical studies, good manufacturing practices, and quality control. To validate a method, it is essential to proceed with the interlaboratory study comparative to the official methodology in force at the moment, in this case, the *in vivo* test described in the Pharmacopeia and carried out by producing and quality control laboratories. For the validation process to stop being an intention and become a reality, investment, reference standards, and integrated action between laboratory professionals and regulatory authorities are necessary. It is necessary to separate the purpose of the trial (pre-clinical, development, quality control) to move forward. This is evident in the important collaborative project between the National Center for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and WHO on why using such a large group of animals for quality control of already licensed products⁷². On the other hand, in preclinical assays, if a single trial is not able to mimic the effects on a whole organism as if it were a living being, consideration should be given to the possibility of using an integrated strategy of more than one *in vitro* trial in place of the *in vivo*¹⁰ trial. Venomics, that is, the study of the proteomics of venoms, can contribute to the identification of the main toxins of the venom and the relationship with the pathophysiology of envenoming. While antivenom detects which venom toxins bind to the antivenom^{42,43,44}. Due to the great world diversity of venoms, it is necessary to carry out unique tests for each country or geographic location, both for pre-clinical tests and for the development of alternative methods⁴³.

The *in vivo* trial does not accurately represent what occurs in snakebites. The main difference lies in the fact that in the *in*

vivo assay, a pre-incubation of venom and antivenom is performed for intraperitoneal inoculation, whereas in accidents, inoculation precedes antivenom therapy, and most people are affected in the lower limbs, but the venom and antivenom mixture is administered intravenous or intraperitoneal to mice. The genetic and biological variability of animals, in addition to the influence of external factors such as environment and stress, is a quality problem that can directly influence the results. For *in vitro* assays, the process of controlling the parameters of environment, temperature, physical space, personnel training and reproducibility is a great advantage, as they have results released in less time and are less expensive than the animal assays. Even so, *in vitro* assays, generally, need to go through a long and expensive process such as validation that comprises five steps and can take about 15 years to complete. Hartung⁶³ highlights some problems that need to be taken into account in cell cultures, but the main point is the implementation in laboratories of good practices in cell culture.

INCQS has a quality assurance system implemented based on ABNT NBR ISO/IEC 17.025:2017⁷³ in order to obtain reliable and accurate results. The implementation of this standard in the routine mitigates most of the problems cited by Hartung⁶³. Therefore, it is essential that the laboratories implement a Quality Assurance system, performing the calibration of the equipment, carrying out the environmental monitoring and maintaining a qualified team, all strictly registered and with easy traceability. Even if the test is not accredited, it is recommended to implement accreditation systems for analysis laboratories, based on the ABNT NBR ISO/IEC 17.025:2017 standard⁷³.

It is worth mentioning that there are other methodologies developed⁶⁸ and pre-validated¹³ that are currently in the validation phase. Although they have not been published, they are based on the property of *Bothrops* venom to cause a cytotoxicity effect on Vero cells and on the property of the *Bothrops* antivenom to inhibit this cytotoxicity. This method aims to be validated for in-process quality control and for regulatory purposes. The criteria defined for acceptance of the assay as valid are venom control and cell control; the cytotoxic effect must show a linear relationship, that is, be proportional to the venom concentrations in at least three consecutive dilutions.

The applicability of the methodology of *in vivo* relative potency was evaluated in a collaborative study with the participation of all national producing laboratories. It has been proposed that the potency of the *Bothrops* antivenom be expressed in neutralizing units (NU) and that each NU corresponds to the ability to 1 mL of *Bothrops* antivenom to neutralize 1 mg of *Bothrops* venom.

As the *in vitro* methodology is more sensitive, it uses a smaller amount of *Bothrops* antivenom compared to the one used *in vivo*, therefore, to make the equivalence of the values between the methodologies we must convert the values to NU^{13,14,68}. Therefore, as the minimum potency for the release of a batch of *Bothrops* antivenom according to the Brazilian Pharmacopeia is 5 mg/mL, this limit would be considered as a minimum of



5 NU, both in the murine system (*in vivo*) and in the cytotoxicity inhibition system (*in vitro*). As Araújo et al.⁶⁶ demonstrated in 2017, the best way to correlate two methods is through the use of well-established and preferably validated reference standards. For assays with venom, it is worth noting that one of the advantages of the *in vitro* method is precisely the economy of reference venom and antivenom.

As part of the validation process of the Vero cell cytotoxicity trial, an *in vitro* trial with enzyme inhibitors was performed.⁷⁴ Orthophenanthroline (O-PHE) inhibited metalloproteases, while p-bromophenacyl bromide (p-BPB) was able to inhibit phospholipases A2, proving that there was inhibition of the cytotoxicity activities of the main enzymes present in the *Bothrops* venom responsible for both the toxicity in humans and lethality in mice.

It is important to highlight that, due to the characteristics of snakebite envenoming, it is necessary to evaluate the biological effect of both the venom and the antivenom. It is worth mentioning that the *in vitro* evaluation of the neurotoxic effects was not part of this research, which, in principle, limits this proposal to snakes that do not have neurotoxicity. Furthermore, due to the complexity of the composition of snake venoms, statistical correlation and proof of the ability to quantify the biological effect of both venoms and antivenoms are essential for the acceptance of the proposed methods.

Of the 31 studies selected in this review, 15 were performed using snake venoms from Brazil, demonstrating that Brazilian researchers are engaged in the search for alternative methods. Despite the expressive numbers, intense action and collaboration between the various institutions is necessary, such as: academic, research, production, and control laboratories in order to promote the necessary advances for the validation of these studies. In addition to being scarce, studies on alternative methods to the use of animals are very segmented. Currently, there is great difficulty in finding data regarding methodologies, official compendia and advances in this area, since there is no compilation of them in official websites^{75,76}.

Through a partnership with the Brazilian National Health Surveillance Agency (Anvisa), the Brazilian Center for the Validation of Alternative Methods (BraCVAM) was created in 2012, pioneer in

Latin America and which aims to validate alternative methods to the use of animals in experimentation and education. The creation of BraCVAM represents an opportunity for the Brazilian scientific community to provide guidance and support for the validation of alternative methods. In the case of antivenoms that are specific in each country and therefore require different tests, this role is essential so that new methodologies have credibility and can be accepted worldwide.

Reviews are necessary to summarize existing data in the area, refine hypotheses and define strategies for innovation and implementation of alternative methodologies. Although we did a relentless search for articles, probably not all articles related to the topic were found.

The results of this study when considered together with the advantages of the *in vitro* assay (reduction of the time to release batches of antivenom, cost reduction, reproducibility, and robustness) and its usefulness in antivenom efficacy tests present itself as a strong candidate for its use as an alternative for the lethality trial in murine.

CONCLUSIONS

It can be concluded that the methodologies identified in the review are based on the toxic properties of venoms correlated with the effects on *Bothrops* accidents. Due to the diversity of toxins found in each genus of snakes, it is understood that it is extremely important that the potency test of antivenoms is based on the evaluation and precise quantification of the inhibition of the biological activity of the venoms. Another important issue when proposing a substitute method is the cut-off point that must be considered when approving the batch.

Methods that are based on cytotoxicity should be prioritized within the strategy of alternative methods, precisely because cytotoxicity assays for the analysis of biological products are simple, low-cost methodologies with successful examples of implementation in quality control laboratories for regulatory purposes. However, it is still necessary that efforts are made in interlaboratory validation tests, allowing the regulatory use and permeability of these tests in the research and production of *Bothrops* antivenoms.

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Author's Contributions

Nundes RNC - Conception, planning (study design), acquisition, analysis, data interpretation, and writing of the work. Araújo HP, Alves GG, Gonzalez MS - Conception, planning (study design), and writing of the work. Delgado IF, OAF Presgrave, Moura WC - Writing of the work. All authors approved the final version of the work.

Conflict of Interests

The authors inform that there is no potential conflict of interest with peers and institutions, politicians, or financial in this study.



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