Received: 2 April 2021 | Revised: 1 June 2021 | Accepted: 3 June 2021

DOI: 10.1002/dta.3108

REVIEW

WILEY

The application of laboratory-based analytical tools and techniques for the quality assessment and improvement of commercial antivenoms used in the treatment of snakebite envenomation

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Funding information
Department of Science and Technology, Ministry of Science and Technology, India, Grant/Award Number: INRC/AISTDF/R&D/P-3/2017

Abstract

Snakebite envenomation is a public health problem of high impact, particularly for the developing world. Antivenom, which contains whole or protease-digested immunoglobulin G, purified from the plasma of hyper-immunized animals (mainly horses), is the mainstay for the treatment of snakebite envenomation. The success of antivenom therapy depends upon its ability to abrogate or reduce the local and systemic toxicity of envenomation. In addition, antivenom administration must be safe for the patients. Therefore, antivenom manufacturers must ensure that these products are effective and safe in the treatment of envenomations. Antivenom efficacy and safety are determined by the physicochemical characteristics of formulations, purity of the immunoglobulin fragments and antibodies, presence of protein aggregates, endotoxin burden, preservative load, and batch to batch variation, as well as on the ability to neutralize the most important toxins of the venoms against which the antivenom is designed. In this context, recent studies have shown that laboratory-based simple analytical techniques, for example, size exclusion chromatography, sodium dodecyl sulphate polyacrylamide gel electrophoresis, mass spectrometry, immunological profiling including immuno-turbidimetry and enzyme-linked immunosorbent assays, Western blotting, immune-chromatographic technique coupled to mass spectrometry analysis, reverse-phase high performance liquid chromatography, spectrofluorometric analysis, in vitro neutralization of venom enzymatic activities, and other methodologies, can be applied for the assessment of antivenom quality, safety, stability, and efficacy. This article reviews the usefulness of different analytical techniques for the quality assessment of commercial antivenoms. It is suggested that these tests should be applied for screening the quality of commercial antivenoms before their preclinical and clinical assessment.

KEYWORDS
adverse reactions, antivenom neutralization, antivenom safety, *in vitro* laboratory assessment, mass spectrometry

1 | THE GLOBAL IMPACT OF SNAKEBITE ENVENOMATION: A NEGLECTED TROPICAL DISEASE FOR DEVELOPING COUNTRIES

Snakebite envenomation is a burning problem in developing countries, largely in tropical and subtropical regions of the world. South and Southeast Asia, sub-Saharan Africa, and Central and South America are the primary contributors to the global snake bite burden; the highest load of snakebites occurs in South and Southeast Asia.[1,2] The data generated from hospital-based epidemiological surveys conducted in different parts of the world have estimated that at least 421,000 snakebite cases occur per year which result into about 20,000 deaths, although the numbers may be as high as 1,841,000 cases and 94,000 fatalities.[1] However, due to the limitation of proper data keeping in hospitals and reporting of snakebite incidence in developing countries, the actual numbers are likely to be much higher.[1] For example, a nationally representative study on causes of mortality in India revealed the occurrence of 45,900 deaths due to snakebites, which is much higher than the official hospital-based records.[3] The same is true for Vietnam.[4] Thus, snakebite envenomation represents a significant portion of the overall global load of neglected tropical diseases.[5]

Snakebite mostly affects impoverished and underserved rural communities. Consequently, it is a "disease of poverty,"[6] which urgently requires renewed attention of national and global health authorities. In many cases, affected people have to cover the expenses of the treatment, thus fueling a vicious cycle that further deepens them into poverty. There has been a growing awareness of the impact of snakebite envenomation on a global basis, which has resulted in the incorporation of this disease in the official list of neglected tropical diseases at the World Health Organization (WHO).[7] Moreover, a resolution on snakebites was adopted by the World Health Assembly in 2018, and the WHO launched a global strategy to control and prevent these envenomations, aimed at reducing by 50% the number of deaths and disabilities due to snakebites by the year 2030.[8] One of the four pillars of this strategy is to provide safe and effective treatments. Therefore, ensuring the manufacture and accessibility of safe and effective antivenoms is one of the main goals of the global efforts to reduce the impact of snakebite envenomation.

2 | ANTIVENOM: BASIC ASPECTS OF MANUFACTURE AND THE PROBLEM OF ADVERSE REACTIONS

The administration of antivenom raised against the venom of a particular species of snake (monospecific antivenom, MAV), against venoms of two species (bispecific antivenom, BAV), or against venoms from more than two species (polyspecific or polyvalent antivenom, PAV) is the cornerstone of the treatment for snakebite envenomations. The venoms used for the production of antivenoms are generally obtained from the snakes of highest medical impact in the countries or regions where the antivenom is distributed. Although the use of monospecific antivenom is generally more efficient and potent to neutralize venom-induced toxicity, the use of monospecific antivenom is usually not feasible due to difficulties for identifying the species responsible of the bite, due to lack of specific commercial diagnostic kit in most regions of the world.[9] Further, because a particular geographical region may be habitat of several species of snakes, in general, PAVs are preferred. For example, in the Indian subcontinent, PAV is produced against the "Big Four" venomous snakes of this region (*Naja naja*, *Daboia russelii*, *Echis carinatus*, and *Bungarus caeruleus*), and this PAV is also used in neighboring countries for the treatment of snakebite envenomation.[10,11]

Antivenom was first used for snakebite treatment by Albert Calmette in 1894.[12] Initially, crude hyperimmune serum was used and, subsequently, steps were introduced to generate purified preparations of immunoglobulins or immunoglobulin fragments. The basic strategy for antivenom production has remained fundamentally the same, although significant improvements in the manufacture methodology have been incorporated.[13] Antivenom production starts by immunizing large animals (usually horses, but some manufacturers use sheep, goats, llamas, or donkeys) with venom from a single species (MAV), two species (BAV) or from various species (PAV) of snakes. Generally, the first injections are done with Freund's complete and incomplete adjuvants, but further immunizations are carried out by using aluminum salts, other adjuvants or plain saline solution.[14] The animals produce neutralizing immunoglobulins progressively against the venom components. When the titer of antivenom IgG in horse plasma reaches its optimum level, as determined by immunoassays or neutralization tests, the animals are bled, and plasma is separated and used for antivenom production by following the fractionation protocols used by manufacturers.

Initially, hyper-immunized horse serum was directly administered to snakebite patients; however, severe adverse reactions to horse serum led to improvements in antivenom production technology.[15] Currently, diverse protocols are followed for the purification of immunoglobulins or immunoglobulin fragments. Most manufacturers refine antivenom by pepsin-digestion of plasma aimed at eliminating the Fc fragment of immunoglobulins, with the generation of F $(ab')_2$ fragments, and also for degrading other plasma proteins, while one manufacturer uses papain digestion to obtain Fab fragments.[16–18] Differential precipitation of plasma proteins is generally achieved by the addition of ammonium salts or caprylic acid.[19,20] Some manufacturers introduce additional steps in plasma fractionation, such as ion-exchange chromatography, and dedicated steps for the removal/inactivation of viruses.[14] IgG, F $(ab')_2$, and Fab antivenoms differ in their pharmacokinetic and pharmacodynamic properties; IgG has lower distribution volume and long half-life, whereas F $(ab')_2$ and Fab antivenoms have higher distribution volumes and shorter half-life.[21–23]

Despite significant improvements in antivenom quality over the last decades, antivenom therapy is not free of limitations. Adverse reactions associated with the intravenous administration of antivenom can be a serious threat for management of snakebite.[24] Figure 1 shows the various types of adverse reactions to antivenom and the possible mechanisms involved. Antivenom reactions in patients can be

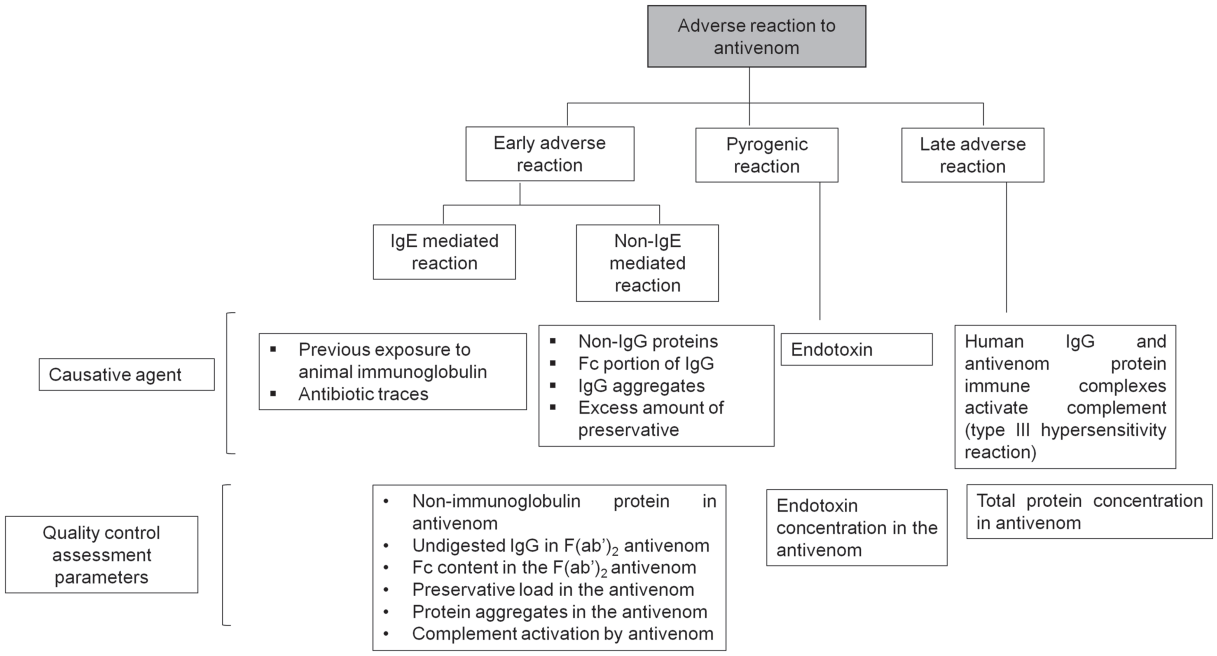

FIGURE 1 Types of adverse reactions generated by antivenom administration, mechanisms involved, and laboratory analyses aimed at predicting the likelihood of reactions

broadly categorized into (i) early adverse reactions, (ii) pyrogenic reactions caused by microbial endotoxins, and (iii) late adverse reactions.

Early adverse reactions to antivenoms, mediated by either IgE or non-IgE-based mechanisms, generally occur after 10–180 min of administration of antivenom and are characterized by cutaneous manifestations (urticaria, itching) or systemic effects (vomiting, fever, nausea, bronchospasm, hypotension, diarrhea, abdominal colic, and tachycardia).[24] IgE-mediated reactions occur in people previously sensitized to equine proteins and result from the degranulation of mast cells and basophils after binding of antivenom proteins to specific IgEs in the membrane of these cells. As a consequence, there is a release of histamine and synthesis of mediators like leukotrienes and cytokines.[25]

The majority of early adverse reactions to antivenom is non-IgE mediated occurring in patients who have no previous medical history of exposure to antivenom. The precise mechanisms behind these de novo reactions have not been established, although it has been proposed that the anti-complementary activity and the presence of heterophilic antibodies present in antivenom or plasma of patients are likely to be involved.[26–30] Activation of complement by antivenoms might be due to the presence of the Fc portion of the whole IgG molecule, protein aggregates, or excessive amounts of preservatives (cresol or phenol).[31–33] Heterophilic antibodies against horse immunoglobulins have been detected in human serum and may be involved in these reactions.[30] The incidence of early adverse reactions to antivenoms greatly varies depending on the products and ranges from 5% to 88%.[25] Pyrogenic reactions also occur in the early time after antivenom treatment and are attributed to the contamination by endotoxins released from the outer membrane of Gram negative bacteria due to contamination during antivenom manufacture.[14] Endotoxin activates macrophages and monocytes to release TNF-α and other cytokines that trigger host responses.[34,35]

Late adverse reactions to antivenom are due to IgG mediated immune-response against the heterologous protein in the antivenom, with the formation of immune aggregates which trigger a typical type III hypersensitivity reaction known as serum sickness, which manifests 5 to 15 days after antivenom infusion.[14,36,37] Clinical studies have shown that about 10–56% antivenom treated patients show late adverse reactions to antivenoms.[36,38,39] The safety of antivenoms is closely related to their physicochemical quality and purity. Therefore, the detailed analysis of antivenom composition and purity is paramount to ensure their suitability for human use.

3 | GENERAL ISSUES RELATED TO THE QUALITY OF ANTIVENOMS

There are 45 antivenom manufacturing laboratories around the globe (complete list of manufacturers are given in the WHO website http://apps.who.int/bloodproducts/snakeantivenoms/database/). Notably, except for several basic procedures of the routine in-house quality control of antivenoms, in many instances, no other laboratory tests (either by the manufacturing laboratory or by regulatory authorities) are done on a regular basis to assess the quality of antivenoms and their batch-to-batch variation in potency, quality (homogeneity of preparation), and safety. This is a serious drawback in the international efforts to improve the quality and safety of antivenom preparations. Moreover, the lack of quality control assessment by the national

regulatory authorities in many countries opens the possibility of introducing unsafe or ineffective antivenoms.

The efficacy of an antivenom greatly depends on the source of snake venoms used for immunization of horses to produce antivenom. Thus, efficacy is affected by the variation in venom composition between the species used in immunization and the species causing the accidents in different geographical settings. Variations may also occur within the same species, owing to geographic and ontogenetic variability.[40–46] The neutralization potency of an antivenom is related to the proportion of venom-specific or toxin-neutralizing antibodies present in the antivenom against a particular venom.[42,47] Neutralization potency and production of specific antibodies is associated to the genetic background of horses and the abundance and immunogenicity of toxins in the venoms.[48] A higher quantity of venom-specific antibodies in a commercial antivenom will enhance its efficacy to neutralize the venom toxins, and consequently, less antivenom vials would be required to counteract the deleterious effects of envenomation.[39] The higher the dose of antivenom required, the higher the load of heterologous proteins administered, and the higher the likelihood of developing adverse reactions.[10,36]

The number of antivenom manufacturers and the volume of the antivenom produced globally are not enough to cover the current needs of antivenoms, especially in the southeast Asia, sub-Saharan Africa, and some parts of Latin America.[49,50] Most of the snakebite-prone regions are developing countries, and they may not be economically sound enough to produce region specific antivenoms; therefore, they rely upon imported antivenom from other countries. For example, in the Indian subcontinent Pakistan, Bangladesh, Nepal, Sri Lanka, Myanmar, and Cambodia are mostly dependent on the PAV manufactured by India.[51–55] Surprisingly, most of the antivenom used in south Asia imported from other countries are not subjected to independent quality and safety assessment. A similar situation occurs in sub-Saharan Africa.[56] Therefore, the preclinical and clinical assessments of several antivenoms distributed in diverse geographical regions have not been well substantiated.[53]

Production of antivenom is a multistep protocol, and each step is crucial for guaranteeing its quality. Thus, any deviation in the production process or non-compliance with Good Manufacturing Practices (GMPs) may end up in substandard products, with the consequences for the patients. Besides efficacy (venom neutralization potency) of the antivenom against the venoms for which it is to be used, the quality of these products involves homogeneity of preparation, fulfillment of required physicochemical standards, and parameters that ensure a good safety profile (for example, stability, sterility, and lack of endotoxin contamination). These parameters must be ensured not only at the manufacturing laboratories but also by the national regulatory agencies.

In addition to the neutralization of lethality test (potency test), which is the gold standard for the preclinical efficacy of antivenoms, the WHO Guidelines for the production and control of antivenoms include several tests for the quality control of these products (Table 1). However, not all antivenom manufacturing laboratories carry out these tests in their routine evaluation of antivenoms, and few regulatory agencies include them when approve an antivenom for clinical use. In addition to these tests, other analyses could greatly expand the portfolio of assays used in the evaluation of antivenoms. Several of these assays are not to be included in the routine analysis of antivenom batches but are useful to assess the quality of antivenoms during the development stages and prior to their introduction to the market. Likewise, these assays are useful for performing an analysis of the quality of existing antivenoms that have not been thoroughly evaluated yet. The following sections discuss some of these tests.

The quality control of antivenoms can be envisaged from a dual perspective: there is a set of assays that manufacturers and quality control laboratories must do for every batch of antivenom that is distributed for clinical use. The WHO has specified which are these mandatory assays for the routine quality control of antivenoms[14] (Table 1). In addition, laboratories working in the development of new antivenoms and the improvement of existing antivenoms should use a variety of laboratory methods, which enable the analysis of the purity, safety, stability, and immunological appropriateness of antivenoms. These methodologies are useful to evaluate aspects in antivenom manufacture such as immunization schemes, venoms selected for immunization, fractionation protocols, and methods introduced to reduce the risk of infectious agents. This will result in the improvement of antivenom manufacturing technologies and, consequently, in the development of safer and more effective products. The methodologies used in the assessment of antivenom quality are described and discussed in this review, with emphasis on *in vitro* methodologies.

4 | LABORATORY-BASED SIMPLE ANALYTICAL TECHNIQUES FOR QUALITY CONTROL OF ANTIVENOM

Physicochemical, biophysical, biochemical, and immunological characterization of antivenoms are easy to perform in the laboratory, since these techniques are cost-effective and are quite useful for addressing the quality, efficacy, stability, and safety of antivenom before their therapeutic application. Some of these methods have been used in the characterization of several antivenoms (Table 2). The following sections describe the laboratory-based analytical techniques that may be used for quality control of antivenom, some of which are included in the WHO guidelines for the production and control of antivenoms.[14]

4.1 | Physicochemical assessment

4.1.1 | Residual moisture, reconstitution, and extractable volume

Antivenoms are marketed as either liquid or freeze-dried products. Freeze-dried antivenoms have advantages since they do not require maintaining the cold chain for storage and transportation. Therefore,

TABLE 1 Parameters that should be assessed in the routine quality control of antivenoms, according to the WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins[14]

Parameters	Testing methods	Requirements
Appearance	Observation of color, cloudiness, and appearance of the powder (in case of freeze-dried preparations)	Compliance with the description of the marketing dossier
Solubility (freeze-dried preparations)	Addition of solvent and observation of time to reach solubility and of appearance	Product should be completely dissolved within 10 min. Solution should not be cloudy
Extractable volume	Extraction of the total volume from the vial or ampoule	Compliance with the indications of the label
Venom neutralizing potency	Determination of the Median Effective Dose (ED_{50}), that is, the ability to neutralize the lethal effect of venom. Expressed as the mg venom neutralized per ml antivenom	Compliance with the specifications of ED_{50} for the product or with the requirements of national regulatory agencies.
Osmolality	Determination of osmolality by accepted methods	At least 240 mOsm/kg
Identity test	Biological, physicochemical or immunological tests	Identity of antivenom confirmed
Protein concentration	Kjeldahl, colorimetric, or spectrophotometric procedures	Not more than 10 g/dl
Purity and integrity of immunoglobulin	SDS-PAGE under reducing and non-reducing conditions	IgG or IgG fragments should comprise more than 90% of the proteins
Molecular size distribution	Size exclusion chromatography (FPLC or HPLC)	Product should consist mainly of monomeric IgG or IgG fragments.
Test for pyrogen substances	▪ Rabbit pyrogen test ▪ Limulus Amebocyte Lysate (LAL) test when validated and approved by national regulatory agency	Accepted limits by the Pharmacopeia in use
Abnormal toxicity test	Intraperitoneal injection and observation of signs of toxicity (not required by some regulatory agencies)	No evidence of toxicity
Sterility test	Filtration through membranes, neutralization (when preservatives are used), and addition to culture media (trypticase soy broth and thioglycolate).	Absence of microbial growth
Concentration of sodium chloride and other excipients	Standard chemical methods	Specifications of Pharmacopeias and regulatory agencies.
Determination of pH	By using a potentiometer (pH meter)	Specifications of Pharmacopeias and regulatory agencies (generally neutral pH).
Concentration of preservatives	Spectrophotometric or HPLC-based methods	Phenol: maximum 2.5 g/L Cresol: maximum 3.5 g/L
Chemical agents used in plasma fractionation	Several methods depending on the chemical agent to be quantified	Specifications of Pharmacopeias, national regulatory agencies or manufacturers.
Residual moisture (freeze-dried preparations)	Karl-Fischer titration, gravimetric or thermogravimetric methods	Less than 3%

freeze-dried antivenom is particularly useful in rural settings of developing countries where often the cold chain cannot be maintained, particularly for storage. Moreover, the shelf-life of freeze-dried antivenoms (3–5 years at room temperature) is more prolonged than that of liquid antivenoms (3 years at 2–8°C). However, the freeze-drying procedure introduces additional costs, and if the process is carried out under non-ideal conditions, the product can be irreversibly damaged. Remaining of moisture can cause denaturation of the product resulting in aggregation of immunoglobulin molecules. Therefore, the determination of residual moisture content by using the Karl Fischer titration method, gravimetric methods, or thermogravimetric methods is recommended for these products.[14,63] The moisture content in freeze-dried antivenoms is recommended to be below 3%[14]; however, residual moisture could be higher for an antivenom

TABLE 2 A summary of the assessment of *in vitro* quality of several commercial antivenoms by different analytical methods

Antivenom	Manufacturer	Assessment of quality	Reference(s)
PoliVal-ICP (polyvalent bothropic, crotalic, lachesic). Formerly known as "polyvalent antivenom"	Instituto Clodomiro Picado, Costa Rica	▪ Electrophoretic and chromatographic profiling ▪ Determination of aggregate content ▪ Anti-complementary activity	Otero-Patiño et al.[57]
Antivipmyn Africa	Instituto Bioclon, Mexico	▪ Total protein determination ▪ Electrophoretic and chromatographic profiling	Ramos-Cerrillo et al.[58]
PoliVal-ICP (Both IgG and F (ab')$_2$ antivenoms)	Instituto Clodomiro Picado, Costa Rica	▪ Total protein determination ▪ Endotoxin load ▪ Preservative (phenol) content Determination of aggregate content ▪ Physicochemical characterization	Otero-Patiño et al.[38]
PoliVal-ICP	Instituto Clodomiro Picado, Costa Rica	▪ Total protein determination ▪ Evaluation of purity of IgG or F (ab')$_2$ antivenoms by FPLC size exclusion chromatography and SDS-PAGE analysis ▪ ELISA determination of antivenom antibodies	Segura et al.[59]
Bothropic, bothropic-crotalic, bothropic-lachesic, crotalic, elapidic (Brazil); PoliVal-ICP, CoRal-ICP, EchiTAb-plus-ICP (Costa Rica)	Instituto Clodomiro Picado, Costa Rica and Instituto Butantan, Brazil	▪ Total protein determination ▪ SDS-PAGE analysis of the antivenom ▪ Complement activation properties by the antivenoms ▪ Immunological binding assay by Western blot	Squaiella-Baptistao et al.[60]
PoliVal-ICP	Instituto Clodomiro Picado, Costa Rica	▪ Endotoxin load in the antivenom	Solano et al.[61]
Anti Bisa Ular antivenom raised against *Naja sputatrix*, *Bungarus fasciatus* and *Calloselasma rhodostoma*	BioFarma Pharmaceuticals, Bandung, Indonesia	▪ Total protein determination ▪ Electrophoretic and chromatographic profiling of antivenom ▪ Mass spectrometry analysis to determine non-immunoglobulin proteins ▪ Immunological binding assay by ELISA	Tan et al.[62]
Snake Venom Antiserum	Bharat Serum and Vaccines Pvt. Ltd., India	▪ Total protein determination ▪ Physicochemical characterization by assessing the appearance, reconstitution time, residual humidity, turbidity and structural analysis by scanning electron microscope	Herrera et al.[63]
Antivipmyn® Fabotherapic Polyvalent Antivenom	Instituto Bioclon, Mexico		
Neuro Polyvalent Snake Antivenin	Queen Saovabha Memorial Institute, Thailand		
Snake Venom Antiserum	VINS Bioproducts Ltd., India		
Fabotherapic Polyvalent Antivenom,	Birmex, Mexico		
Polyspecific Antivenom	Instituto Clodomiro Picado, Costa Rica		
Sii Polyvalent Anti-snake Venom Serum	Serum Institute of India Ltd., India		
Combipack snake venom antiserums (Pan Africa)	Premium Serum and Vaccines Pvt. Ltd., India	▪ Total protein determination ▪ Electrophoretic and chromatographic profiling ▪ Mass spectrometry analysis to determine non-immunoglobulin proteins ▪ IgA/IgE co-separation ▪ Endotoxin load ▪ Preservative (m-cresol) content ▪ Immunological profiling by ELISA and Western blot analysis Determination of *in vivo* acute toxicity	Patra et al.[64]

(Continues)

TABLE 2 (Continued)

Antivenom	Manufacturer	Assessment of quality	Reference(s)
Snake Venom Antiserum	Bharat Serum and Vaccines Pvt. Ltd., India	■ Total protein determination ■ Electrophoretic and chromatographic profiling ■ Mass spectrometry analysis to determine non-immunoglobulin proteins ■ Determination of IgG content by IgG specific antibody ■ IgA/IgE co-separation ■ Endotoxin load ■ Preservative (m-cresol) content ■ Determination of venom–antivenom binding affinity by spectrofluorometric titration analysis ■ Determination of venom specific antibody from spectrofluorometric titration curve	Patra et al.[47]
Snake Venom Antiserum	Premium Serum and Vaccines Pvt. Ltd., India		
VI – ASV	Virchow Biotech Pvt. Ltd., India		
Snake Venom Antiserum	Vins BioProducts Limited, India		
Snake Antivenin (Polyvalent) IP	Biological E Limited., India		
CSL antivenoms (Brown snake; Tiger snake; Black snake; Death adder)	CSL Ltd	■ Immunoturbidimetry assay to detect venom–antivenom binding	O'Leary et al.[65]
EchiTAb-Plus-ICP® IgG	Instituto Clodomiro Picado, Costa Rica	■ Determination of venom specific antibody by third generation antivenomics approach ■ Venom–antivenom binding by size exclusion chromatography	Pla et al.[66]
Commercial equine bothropic antivenom	Instituto Butantan, São Paulo, SP, Brazil	■ *In vitro* neutralization of enzymatic activities ■ Venom–antivenom binding by size exclusion chromatography	Rita de Cássia[67]
ANAVIP® (i.e., Anavipmyn)	Rare Disease Therapeutics, Inc., USA	■ Venom–antivenom binding by size exclusion chromatography	Tanner et al.[68]
CroFAB® (Crotalidae Polyvalent Immune Fab [Ovine])	Protherics Inc., USA	■ Venom–antivenom binding by size exclusion chromatography	Sanny[69]

formulation if this parameter is supported by the manufacturer with the corresponding stability tests. The quality of freeze-dried antivenoms can be further analyzed by using scanning electron microscopy (SEM).[63] Moreover, the glass transition temperature and the collapse temperature, which are relevant parameters during the design and storage of freeze-dried products, can be assessed by differential scanning calorimetry (DSC) and freeze-drying microscopy (FDM).[70]

Freeze-dried antivenoms should be readily dissolved in the volume of solvent, usually water for injection or saline solution, as indicated by the manufacturer. Once the solvent is added and the preparation gently mixed, the product should be dissolved in less than 10 min.[14] Moreover, the solution should be free of particles and should not be cloudy. The same criteria hold for liquid antivenoms in terms of appearance. The extractable volume for antivenom vials should comply with the specifications indicated in the label, for example, 10 ml.

4.1.2 | Determination of pH

The pH of antivenom solutions, in both liquid and freeze-dried preparations, should be in the range of 7.0–7.2.[14] Formulation of the antivenom at pH more than 7.5 is not recommended as higher pH affects the stability of antivenom proteins and favors formation of immunoglobulin aggregates that leads to complement activation.[32] Some antivenom manufacturers prefer to maintain the pH towards slightly acidic values to reduce aggregate formation.[14] Formulation of antivenoms at low pH has been studied with good results as a viral inactivation step without affecting the neutralizing potency. However, an increase in immunoglobulin aggregates was observed.[71] F $(ab')_2$ antivenoms are prepared by digestion with pepsin at pH 3.0–3.5, and then the pH is adjusted to 7.0–7.2 with NaOH. For quality assurance, the pH of the reconstituted antivenom can be measured with a potentiometer, which is a simple technique.[14]

4.1.3 | Estimation of preservative content

Addition of preservative in the antivenom preparation is generally used to avoid contamination for long term storage. For example, m-cresol, phenol, and thimerosal are commonly used preservatives in antivenom formulation,[14] although thimerosal and other mercury-containing preservatives are no longer recommended in antivenom manufacture. The maximum allowed concentrations of these

preservatives are 2.5 g/L for phenol and 3.5 g/L for cresols.[14] Excess amount of preservative in the antivenom preparation may increase the formation of dimers or aggregates of IgG or F (ab')$_2$ molecules which leads to complement activation.[32] Freeze-dried antivenoms do not require the addition of preservatives because contamination during storage is limited by the low content of moisture, and usually bacteriostatic compounds like phenols are removed during the freeze-drying step. Estimation of preservative concentration can be done in the laboratory by reversed-phase high performance liquid chromatography (RP-HPLC) analysis.[14] The RP-HPLC analysis of m-cresol can be performed on a C_{18} RP-HPLC column (the diameter and pore size of the column depend upon the availability in the laboratory) using a mobile phase containing 0.1% trifluoroacetic acid (TFA) in 60% methanol with an isocratic program. The detection is carried out in 217 nm.[72] The preservative concentration can be determined from the HPLC standard curve of this compound run under identical chromatographic conditions.[47,64,72] The standard curve used for estimation of m-cresol or phenol content should be accurate, linear, and validated. Spectrophotometric methods can also be used, such as the procedure of 4-amino antipyrine for the quantification of phenol.[14]

4.1.4 | Concentration of protein, excipients, and of substances used during plasma fractionation

Antivenoms are composed of immunoglobulins or their fragments F (ab')$_2$ or Fab. Total protein concentration in antivenoms can be assessed by various methods.[14] Protein concentration is adjusted as to achieve the accepted neutralizing potency values; thus, the protein concentration in antivenom preparations varies between products and between batches in the same product. A protein concentration no higher than 100 g/L is the accepted value.[14] Protein concentration can be quantified by several methods, such as Kjeldahl, Biuret, or other colorimetric procedures.[14] Antivenoms are formulated with excipients, such as sodium chloride, to guarantee the adequate osmolality of the product and, in some cases, with sucrose and other stabilizers. The content of these excipients should be determined to assess whether the product fulfills the requirements. It is recommended that the osmolality of antivenom preparations should be at least 240 mOsm/kg.[14] Likewise, the concentration of reagents used during plasma fractionation, such as ammonium sulphate, caprylic acid, pepsin, or papain, should be determined, since these compounds should be eliminated in the final product. Thus, maximum values acceptable for these molecules should be guaranteed.[14] Caprylic acid can be quantified by a validated protocol using RP-HPLC with ultraviolet detection.[73] This chromatographic technique is commonly available in current quality control laboratories.

4.1.5 | Assessment of purity and aggregate content

The assessment of the purity of the immunoglobulins or their fragments is one of the most important aspects in the analysis of antivenoms that contain either immunoglobulins or its digested products, F (ab')$_2$ or F (ab). Antivenoms may contain impurities resulting from inadequate fractionation protocols, especially albumin and other non-IgG plasma proteins.[47,64] In addition, poor enzymatic digestion of IgG molecules results in retaining its Fc portion in F (ab')$_2$ or F (ab) preparations, which could be related to adverse reactions when administered in patients.[74,75] Moreover, antivenoms might contain aggregates of immunoglobulins and other plasma proteins that impair their quality. The purity of immunoglobulin fraction (IgG and/or its pepsin or papain digested product) in antivenoms can be evaluated by size exclusion chromatography (SEC) (also sometime referred as gel filtration chromatography) and/or SDS-PAGE analysis, which can be readily performed in the laboratory, as shown by several studies carried out with different antivenoms.[17,47,58,59,62,64]

SDS-PAGE analysis, run under reducing or non-reducing conditions, separates proteins according to their molecular mass. The purity and homogeneity of the immunoglobulin fractions of antivenom preparations (IgG, F (ab')$_2$, or Fab) can be determined on the basis of their corresponding molecular masses (IgG, 150 kDa; F (ab')$_2$, 100 kDa; Fab, 50 kDa).[14] The quantification of the immunoglobulin fractions (IgG, F (ab')$_2$, or Fab) can be determined by densitometry analysis of SDS-PAGE bands of antivenom when compared with the appropriate control of the purified active component.[47,62–64]

The homogeneity and purity of the immunoglobulins or their fragments in antivenom preparation can also be determined by SEC. This procedure separates the proteins according to their molecular mass, with high molecular mass proteins eluting earlier than low molecular mass proteins. The elution pattern of antivenom subjected to SEC can be compared to the elution pattern of purified IgG/F (ab')$_2$/Fab proteins (run under identical conditions). The identity and molecular mass of immunoglobulins or their fragments can be also confirmed using a SEC calibration kit, with a set of well-defined protein standards. Moreover, the purity of the antivenom can be confirmed and quantified from the area under the peak in the chromatogram.[47,62,64,76] SEC also allows the detection of immunoglobulin dimers, trimers or high molecular mass soluble protein aggregates.

When evaluated by these biophysical techniques, three different batches of PAN Africa PAV (F (ab')$_2$) manufactured by PSVPL against venoms of seven Viperidae and seven Elapidae snake venoms showed 80–90% purity.[64] The PAV raised against venoms of *Bothrops asper*, *Crotalus simus*, and *Lachesis stenophrys* produced by Instituto Clodomiro Picado, Costa Rica, contain 90–93% of F (ab')$_2$ and whole IgG molecules.[59] A recent study using these techniques has shown that the PAV used in Indian subcontinent raised against the "Big Four" venomous snakes of India have 75–90% purity of F (ab')$_2$ molecules.[47] It is necessary to do this type of assessment to other antivenoms from various countries.

Aggregation of therapeutic antibodies due to inadequate fractionation protocols or storage conditions of the product, a deficient freeze-drying process, or excessive moisture in case of freeze dried products is a well-known phenomenon[77] and results in alteration of solubility, development of turbidity (insoluble aggregates), and loss of the therapeutic activity of antivenom.[14,78] Therefore, aggregation

of immunoglobulin molecules preparation should be determined and quantified in every batch of antivenom. WHO has suggested that the antivenom manufacturer should disclose the aggregate content in the final preparation of antivenom.[14] The aggregation of immunoglobulin molecules and their fragments (IgG, Fab, and F $(ab')_2$) can be assessed by simple analytical techniques such as by SEC and/or by SDS-PAGE analysis.[47,62,63,76,79] In SEC, the presence of soluble aggregates is evidenced by a high molecular mass protein peak eluting earlier than the major peak of active molecules (IgG/F $(ab')_2$/Fab).[47] The SDS-PAGE analysis of antivenom under non-reducing conditions can also be used to determine the occurrence of high molecular mass protein aggregates.[47,59,62] However, SDS-PAGE separation under reducing conditions is not recommended because in these conditions there is solubilization of protein aggregates.[80,81] Therefore, SDS-PAGE analysis (under non-reducing conditions) can be used as a complementary assay along with SEC. Assessing of SABU (Serum Anti Bisa Ular) antivenom from Indonesia by SEC showed the presence of 7.3% dimers or aggregates of immunoglobulins.[62]The PAVs from Indian subcontinent showed low to moderate levels of protein aggregates (10–30%) in their preparation as determined by SDS-PAGE analysis and SEC.[47]

Dynamic light scattering (DLS) is another biophysical technique that could be used to determine the occurrence of aggregates of antibodies in antivenom.[82,83] DLS method is based on the principle that freely diffusing material randomly move due to Brownian motion and rapid fluctuations in scattered laser light occur. These fluctuations in scattered light can be directly correlated to the motion of molecules. The signal generated from the scattered intensity from the laser light is acquired and transformed into an autocorrelation function. Therefore, the higher the particle size distribution, the higher the intensity. The DLS method is useful to analyze the high order aggregates because of higher intensity of larger particle.[84] Several studies have determined the aggregate content of therapeutic monoclonal antibodies by DLS method[85–87]; nevertheless, aggregate content analysis in snake antivenom by using this analytical technique is yet to be introduced in the field.

4.1.6 | Mass spectrometry analysis for the assessment of quality of antivenom preparation

During precipitation and/or separation of IgG molecules or fragments from hyperimmune horse plasma, there is a possibility of co-purification of non-immunoglobulin proteins such as plasma albumin, ceruloplasmin and fibrinogen, and undesirable immunoglobulins, for example, IgA, along with the IgG antibodies.[14] The physicochemical purity of antivenom is generally assessed by using chromatographic and SEC techniques, and they basically provide the profile of the antivenom based on the molecular mass of proteins. However, these techniques cannot identify and quantify contaminating plasma proteins having similar molecular masses of immunoglobulins and their fragments in an antivenom preparation. Furthermore, low abundance or degraded proteins in the antivenom may not be detected by chromatographic and electrophoretic techniques. The introduction of the proteomics strategy utilizing high resolution mass spectrometry (such as LC/MS–MS analysis) overcomes this limitation. Mass spectrometry analysis, which is a powerful technique that detects and quantifies a trace quantity of protein, can be applied to assess the purity of therapeutic antibodies and the identification of non-IgG proteins present in the antivenom preparation.[47,62,64,76]

Mass spectrometry provides the molecular mass of a protein or a protein fragment and reveals structural information such as amino acid sequence or the type of post-translational modifications (PTM) that allows the identification of the protein. For the determination of amino acid sequences and PTM, the protein is digested with trypsin to generate peptide fragments, the digested peptides are subjected to nano-HPLC, and the eluted peptides are ionized and transferred to Q-TOF mass spectrometer. The signals from the peptides are assigned to spectra according to their m/z ratio. The raw data generated from mass spectrometry are searched against NCBI or Uniprot non-redundant *Equus caballus* (taxid 9796) database by using data processing software (Peaks Studio/Proteome Discover/Morpheus). The identified proteins are inferred manually and grouped based on their database nomenclature. The relative abundance of the protein can be calculated using MS1-based or MS2-based label free quantification method.[47,64,76] In proteomic analysis, the fragment ion spectra generated are used for the identification of the amino acid sequence of specific peptides; MS1 is usually used for single-stage MS analysis, whereas MS2 is generally used for tandem MS analysis. In MS1, ionization of peptides results in formation of different peaks containing diverse ionized species of different mass-to-charge ratio (m/z). Only the selected ions of a particular *m/z* ratio are further fragmented by allowing them to enter in a collision cell, for example, by collision with a neutral gas molecule, a process known as collision-induced-dissociation. A second mass spectrometer (MS2) separates these smaller fragments according to their *m*/z, and subsequently, they are detected by the detector.

Quantitative strategy in mass spectrometry analysis is based on the fact that intensity in ESI-MS is directly proportional to the concentration of the ions detected.[88] Although this platform has been questioned as a quantitative tool based on instrumental-related and sample-related issues,[89] high precision and accurate results have been obtained with this methodology.[90,91]

Recently, tandem mass spectrometry analysis of a PAN Africa antivenom demonstrated that the three batches of antivenom under testing are composed primarily of immunoglobulins with minor amounts (~7%) of other plasma proteins like albumin, fibrinogen, haptoglobin, alpha-2 macroglobulin, and fibronectin.[64] Quality assessment of SABU antivenom from Indonesia also described the presence of other plasma proteins in trace amounts along with the immunoglobulin molecule.[62] A recent study has also determined and quantified the non-IgG contaminating proteins (ranging from 6 to 20%) in the commercial PAV produced in India.[47] It has been shown that antivenoms from south-East Asia and Indian subcontinent contain other serum proteins from horses (ranging from 8 to 15%).[76] This type of analysis is highly helpful for antivenom manufacturers in order to improve their fractionation protocols aiming at obtaining more purified antivenom preparations.

4.2 | Assays to assess microbial contamination

4.2.1 | Sterility test

Antivenoms should be sterile, that is, free from bacterial and fungal contamination. This is assessed in the routine quality control of antivenoms by testing the product in culture media such as trypticase soy broth and a medium with thioglycolate, following methodologies mentioned in different Pharmacopoeias.[14] When antivenoms contain preservatives, they have to be neutralized before the sterility test is done.

4.2.2 | Determination of bacterial endotoxin contamination

If GMPs are not properly followed during antivenom manufacture, horse plasma or intermediate products along the fractionation line can be contaminated with bacteria, resulting in the presence of bacterial products, such as lipopolysaccharides (LPS, endotoxins) in the case of Gram negative bacteria. The chemicals, raw materials, and glassware used in the manufacturing process may also be the sources of endotoxin contamination unless proper control and depyrogenation are performed.[92] Endotoxins are not removed during the sterilization process. Traditionally, the detection of endotoxin contamination is done by the rabbit pyrogen test, as described in several Pharmacopeias.[14] In order to avoid the use of animals, *in vitro* tests for the detection and quantification of endotoxins have been developed, such as the Limulus Amebocyte Lysate (LAL) assay.[47,61,64] The principle of this test relies on the process of coagulation of the hemolymph of *Limulus* crab or horseshoe crab (*Limulus polyphemus*) in the presence of LPS present in the products. A small amount of LPS activates the coagulation factor present in the hemolymph of *Limulus*, and the reaction can be detected either by coagulation of the preparation or by the colorimetric estimation by a chromogenic substrate. Many commercial endotoxin quantification kits are available, which are easy to perform and give results within 30 min.[93,94]

4.2.3 | Assessment of viral safety of antivenoms

Although there is no report on viral transmission though equine antivenom to humans, the WHO has issued recommendations on ways to ensure the viral safety of antivenoms, by introducing dedicated steps during the fractionation process that reduce the viral load. The viruses present in the horses and their possible status to infect humans are enlisted in the WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins.[14] Viral contamination in antivenom can be assessed by analyzing genetic material (DNA or RNA) of model viruses enlisted in the WHO protocol.[14] Even though analysis of viral contamination is not done on a routine basis in antivenom preparations, manufacturers must ensure that their fractionation protocols include at least two steps of virus removal or inactivation (caprylic acid treatment/acid pH treatment/filtration/pasteurization) and therefore must validate their procedures using available protocols.[14,95]

The *in vitro* evaluation of viral removal/inactivation by the various steps in the fractionation scheme can be carried out in a down-scale approach by the inoculation ("spiking") of hyper-immune plasma, or intermediate products in the fractionation line, with model viruses, followed by the implementation of the fractionation procedures aimed at removing/inactivating viruses. The reduction in the viral load is then assessed by using various susceptible indicator cell lines and is expressed as the log reduction in infectivity. Manufacturers should associate with laboratories with expertise in this type of analysis in order to validate their protocols in terms of viral safety.

The presence of viral DNA or RNA in antivenoms or hyper-immune plasma can be assessed by quantitative PCR.[96] The use of sequential quantitative PCR assays for viral detection is very helpful to identify false-positive results affected by unintended contamination of samples with traces of viral nucleic acids or PCR products.[97] Hitherto, no study has been reported which described the viral load in the antivenom preparations.

4.3 | Anti-complementary activity of antivenoms

IgG is assumed to activate complement pathways by classical, alternative, and lectin pathways.[98] Activation of complement pathway leads to release of anaphylatoxins, which are responsible for mast cell degranulation and the consequent effects.[74,75] The Fc portion of the IgG is mainly responsible for the activation of complement; however, F $(ab')_2$ and their aggregates can also activate the complement pathways.[29,60] Henceforth, quantification of anti-complementary activity of antivenoms, that is, their ability to activate the complement system, is a useful test when assessing the quality of antivenoms. The complement activation by different pathways can be evaluated by *in vitro* laboratory assays by determining the residual hemolytic activity of normal human serum incubated with antivenom, with the use of sheep or rabbit erythrocytes and anti-erythrocyte antibodies, which is a well standardized protocol.[60,99]

The assay for the anti-complementary activity by the antivenom is essential and associated with the several factors like composition of antivenom, presence of aggregates, and excess amount of preservative. However, no reference value has been established for the anti-complementary activity of antivenoms. Therefore, the safety of antivenom may not always be concluded from the results of anti-complementary activity assay. Further preclinical and clinical studies should be designed to determine the reference threshold value for anti-complementary activity of antivenom.

4.4 | Immunological characterization of antivenoms

4.4.1 | Assessment of presence of immunoglobulins other than IgG in antivenom by immunological profiling

IgG or its proteolytically derived fragments are the active substances of antivenoms. Various subclasses of IgGs have the capacity to neutralize venom components.[100] During the fractionation of horse

hyperimmune plasma by non-chromatographic methods, such as those routinely used in antivenom manufacture, there is always a chance of co-precipitation of immunoglobulin isotypes other than IgG, for example, IgA, IgE, and/or IgM, in the antivenom preparation, which have no role in venom neutralization. A small quantity of these non-IgG may not affect the quality of antivenom; however, higher quantities may be detrimental and may contribute to the adverse reactions to antivenom treated patients.[24] Contamination of IgA, IgE, or IgM can easily be detected and quantified by immunological cross-reactivity between antivenom and anti-horse IgA/IgE/IgM specific antibodies by ELISA or Western blot analysis.[47,64]

A recent analysis has shown that PAN Africa country-specific PAV manufactured by several companies in India contain IgA, but not IgE.[47,64] Since IgA has no role in neutralization of snake venom induced toxicity, therefore, co-precipitation of IgA with IgG may decrease the content of therapeutic antibodies in the commercial antivenom.

4.4.2 | Assessment of venom–antivenom immunological cross-reactivity

Immuno-turbidimetry assay

Immuno-turbidimetry assay is based on the principle of immunoprecipitation method where antigen and antibody bind and form large insoluble immunocomplexes. The level of antigen–antibody binding can be measured by the increased turbidity of the solution.[101] Immunoprecipitation is a well-established method to determine the interaction of venom components (antigen) with antivenom (antibodies).[102,103] This preliminary assay has been used to monitor the interaction between venom and antivenom to determine the kinetics in the formation of immunocomplexes. In case of *Bothrops asper* venom and a polyspecific antivenom, it was observed that the turbidity continued to increase over the 30-min incubation period.[104] The binding of antivenom antibodies to venom proteins can also be evaluated in vitro by measuring the absorbance at 340 nm.[65] This turbidity test can be an easy, cost-effective, and rapid method to characterize interaction between venom and antivenom, albeit formation of insoluble immunocomplexes does not always reflect the linearity of neutralization of venom toxins by antivenom, which is the major limitation of this approach.[65]

ELISA and Western blot

The gold standard to assess whether an antivenom is effective against a particular snake venom is the analysis of the neutralization of the lethal activity of the venom in pre-clinical testing. In addition, depending on the toxicological profile of the venom, the neutralization of other toxic activities, for example, hemorrhagic, *in vitro* coagulant, defibrinogenating, and myotoxic activities, should be evaluated.[14] However, in order to reduce *in vivo* mouse experiments, along the 3Rs philosophy[14,105,106] (Figure 2), the *in vitro* screening tests based on immunochemical methods, such as enzyme immunoassays (ELISA) and Western blot, can be used in antivenom manufacturing and quality control laboratories.[43,107–109]

Assessment of venom–antivenom cross-reactivity by ELISA is used to determine the antibody titer against a particular venom or purified toxins. Geographical and species-specific variation in venom composition of snake is a well-known phenomenon.[45,110,111] Therefore, ELISA is a valuable tool to assess whether the antibody titer in antivenom varies when tested against venoms of different geographical origins,[42,43,109] as well as to assess variations in antivenom titers between batches. For example, immunological profiling of *D. ruselii* venom from different geographical regions of India by ELISA and Western blot analyses showed differential cross-reactivity towards commercial PAV, which may be attributed to variation in venom antigenicity due to geographical differences.[41,43] Determination of EC_{50} value (defined as the concentration of the antivenom in which 50% of the venom antigens are bound) by ELISA can be used as proxy to assess the cross-reactivity of antivenoms against venoms.[43,112,113] Moreover, when the toxins responsible for toxicity in a particular venom are identified, through the estimation of the Toxicity Score,[114] it is feasible to develop ELISAs using purified toxins instead of crude venom.

The correlation of ELISA and the lethality neutralization test has provided variable results depending on the venom. As an *in vitro* test, ELISA provides information about immune-reactivity but does not necessarily about neutralization of toxicity. For some venoms, a significant correlation has been described, whereas for others no such correlation exists. Therefore, this analysis must be made on a case-by-case basis and additional validation studies are essential prior to using ELISA as an alternative method for determination of efficacy and quality control of commercial antivenoms.

Western blot analysis of recognition of venom proteins by antivenom antibodies can also be useful to portray the ability to determine whether or not antivenom can immuno-recognize a particular mass range of proteins (toxins). Western blot analysis of Elapidae snake venom proteins has shown that, in general terms, low molecular mass proteins (such as three-finger toxins) are not well immuno-recognized by commercial antivenoms.[109,112,113,115] In contrast, high molecular mass (>45 kDa) venom proteins (e.g., L-amino acid oxidases, metalloproteinases, and phosphodiesterases) are better immuno-recognized than the low molecular mass components of venom (e.g., phospholipases A_2 (PLA_2), C-type lectin-like proteins, disintegrins.[108,116–118] In contrast to ELISA, Western blot analysis only provides a qualitative assessment of antivenom immune-reactivity but is a useful tool to identify venom components poorly recognized by antivenoms.

Size-exclusion chromatography (SEC)

Size exclusion chromatography (SEC) is also a simple, easy to perform method that has long been used for determining antigen–antibody interaction,[119,120] including snake venom and antivenom (Table 2), by incubating them at 37°C for 1 h and then separating the mixture by SEC.[66–69,121] The elution profile of venom–antivenom mixture is compared with those of venom and antivenom alone, where no antigen–antibody interaction occurs ("null profile") and the corresponding areas of the peaks in the chromatogram are

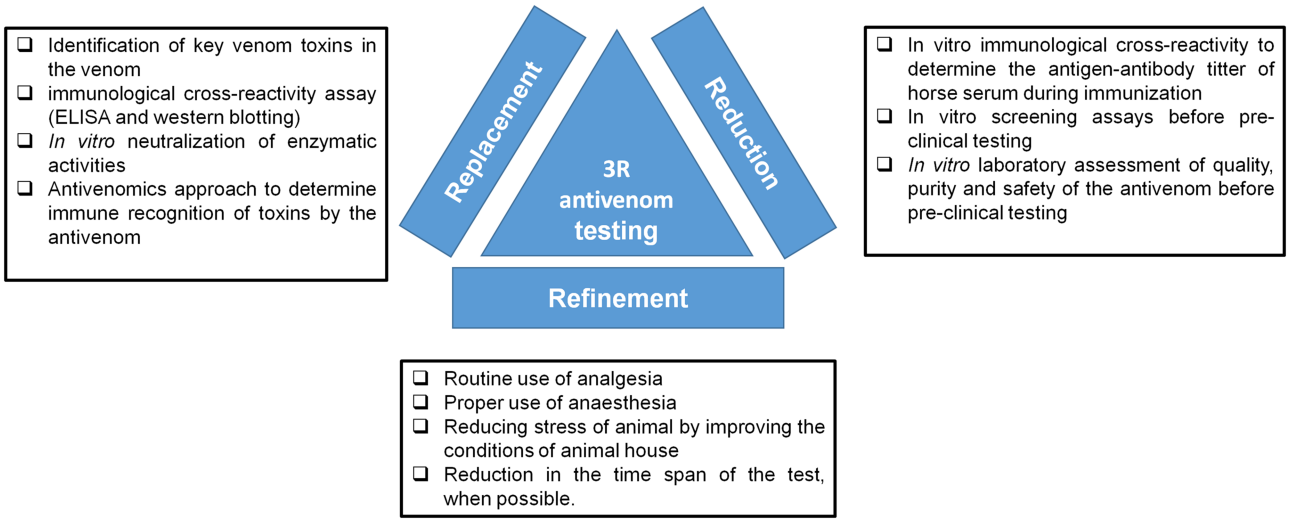

FIGURE 2 Possible approaches to implement the 3Rs (Replacement, Reduction, and Refinement) for antivenom testing[105,106]

determined. Elution profile region areas (areas under the curve) are calculated, and relative venom–antivenom binding capacity is determined based on differences between reaction and null profile region areas.[68,69] The determination of venom–antivenom binding by SEC provides a preliminary idea about the venom binding capacity of antivenom and therefore, may be used as a preliminary screening assay prior to *in vivo* pre-clinical studies. Nevertheless, this method also has limitations since venom–antivenom binding does not always reflect *in vivo* venom neutralization potency of the antivenom. Therefore, this method should be validated by studying its correlation with the *in vivo* neutralization of lethality and toxic activities by commercial antivenoms.[120]

Immuno-affinity chromatography coupled with mass spectrometry analysis

Immuno-recognition studies by the methods previously described are unable to pinpoint in detail the particular venom toxins that escape the immuno-recognition by commercial antivenoms. A significant step forward was the introduction of mass spectrometry-based methods to assess venom composition ("venomics") and antivenom immunoreactivity ("antivenomics").[118,122] Antivenomics methodologies not only allow the identification of recognized and non-recognized venom proteins by an antivenom but also quantify the amounts of antibodies against particular toxins. Initially, immunoprecipitation method was used to precipitate the immunoreactive proteins, whereas poorly or non-immuno-recognized proteins remain in solution, a methodology termed as "first generation antivenomics" approach. These toxins were subsequently identified by LC/MS–MS analysis.[118,123,124]

Subsequent improvement has been made in the identification of poorly immunogenic toxin(s) by a method known as "second generation antivenomics," which is a modification of immuno-affinity chromatography. In this method, antivenom is immobilized in a NHS-Sepharose matrix followed by immunocapturing of venom proteins (both toxins and non-toxic components) by the antivenom coupled to the matrix. Poorly immunogenic venom proteins that are not bound/partially bound to the antivenom are eluted as unbound fractions. Immuno-recognized toxins are then eluted as bound fraction. Both the bound and unbound fractions are separated by RP-HPLC, and their chromatograms are compared with those of native venom, which is followed by LC/MS–MS analysis to identify the immuno-recognized and poorly recognized venom components.[125] For a homologous venom system, information concerning the immunogenicity of venom proteins can be deduced from the results. In the case of a heterologous system (when a venom not included in the immunizing mixture is tested), conclusions about cross-reactivity of the antibodies can be drawn.[126]

A modified second generation antivenomics approach has been described recently where antivenom bound (strongly immunogenic) and unbound venom toxins (poorly immunogenic) are first separated through SDS-PAGE followed by LC/MS–MS analysis of the protein bands to identify and quantify the unbound proteins.[107,113,117] This knowledge is crucial for the improvement of the immunization protocols to generate more effective antivenoms. Therefore, antivenomics analysis should be applied to ensure the quality of the antivenom prior to *in vivo* pre-clinical studies of antivenom.[14] When carrying out antivenomics analyses, several aspects should be considered that might affect the results, such as ligand density, binding and elution conditions, coupling efficiency, amount of antivenom and venom loaded in the column, and the relative abundance of venom proteins.[126] These parameters should be standardized when running these methodologies.

Although this is a robust method for quality assessment of antivenom, not all laboratories have the conditions to carry out these analyses. This calls for the development of partnerships between laboratories working in the development and manufacture of antivenoms and those with mass spectrometry facilities within a cooperation frameshift.

4.4.3 | Determination of toxin-specific antibodies in antivenoms

Determination of toxin-specific antibody in antivenom, especially against the most relevant toxins of a venom, will provide a better insight about the quality and efficacy of the antivenom. Antivenoms may contain non-venom specific antibodies along with the venom specific antibodies. Thus, the higher the proportion of venom/toxin-specific antibodies in commercial antivenoms, the higher the neutralizing efficacy. Therefore, immunization strategies that enable a higher generation of neutralizing antibodies will result in improved antivenoms, since the total amount of protein needed to neutralize the venom would be reduced, with the consequent reduction in the likelihood of adverse reactions.

Venom-specific antibodies in an antivenom preparation can also be determined from the "third generation antivenomics" approach,[66] which was introduced to improve the analytical capability of the immuno-affinity second-generation antivenomics, by incorporating the determination of the maximal binding capacity of an antivenom toward the different proteins present in a venom. This new method can also quantify the fraction of venom-specific antibodies present in a given antivenom.[66] The venom-specific antibody content in a vial can be calculated using the following formula.[42,127,128]

$$\begin{aligned}&\textit{Toxin specific antibody in a vial}\\&(1/2\,\textit{maximal amount}\,(\textit{in}\,\mu\textit{moles})\,\textit{of total venom proteins bound per antivenom vial})\times\\&=\frac{\text{molecular mass (in kDa) of antibody (IgG,160 kDa or F(ab')2,100 kDa) molecule)}}{\text{total amount of antibody (IgG or F(ab')2) (in mg) per antivenom vial}}\end{aligned}$$

Using this protocol, the percentage of venom-specific antibodies in a monospecific *Vipera berus berus* antivenom manufactured in Russia and a monospecific antivenom against *D. siamensis* manufactured in Taiwan was found to be 6.24% and 8.9%, respectively.[127,128] Other studies have applied the third generation antivenomics to the analysis of other antivenoms from Latin America,[129] Africa,[66] and Asia.[42]

4.4.4 | Spectrofluorometric method for assessing venom–antivenom interactions

A recent study from one of our laboratories for the first time has demonstrated the usefulness of spectrofluorometric titration of venom–antivenom interactions to determine the venom-specific antibodies in commercial Indian antivenoms.[47] Proteins have endogenous fluorophores such as tryptophan residues and heme or flavin groups. Protein–protein interaction can be evaluated by monitoring changes in the tryptophan environment detected by changes in its intrinsic fluorescence. When protein–protein interaction occurs, the complex formation leads to changes in the fluorescence emission spectrum either due to a shift in the wavelength of maximum fluorescence emission or to a shift in fluorescence intensity caused by the mixing of two proteins. The proteins are excited at 280 nm, and emission wavelength is monitored from 290 to 500 nm. The strength of venom–antivenom interaction can be determined by calculating the dissociation constant (K_d value), plotting the change in λmax against the concentration of the antivenom (mg protein/ml) using one site-specific binding model of the interactions.[47]

From the one site binding spectrofluorometric titration curve of antivenom–venom interaction, the amount of antivenom that shows saturation in the binding to a fixed amount of a test venom sample (when no further increase in fluorescence signal of venom–antivenom mixture is observed) can be determined. The total amount of immunoglobulin (IgG and/or F $(ab')_2$) in a vial is then calculated from the amount of immunoglobulin molecules present per g protein (determined by LC/MS–MS analysis data). The venom-specific antibodies in PAV (w/w) are determined by using the following formulas[47]

A. $$\begin{aligned}&\textit{Total venom}-\textit{specfic antibodies in a vial}\,(\textit{mg})\\&=\frac{\textit{Amount of venom used in spectrofluorometric titration}(\textit{mg})}{\textit{Amount of PAV in which shows saturation with the venom}\,(\textit{mg})}\\&\quad\times\textit{Total amount of immunoglobulin content}\\&\quad\left[\textit{IgG and/or F}\left(ab'\right)2\right]\ \textit{in a vial}\,(\textit{mg})\end{aligned}$$

B. $$\begin{aligned}&\textit{Percentage of venom}-\textit{specfic antibodies in a vial}\\&=\frac{\textit{Total venom}-\textit{specific antibodies in a vial}\,(\text{mg})}{\textit{Total immunoglobulin content}}\times 100\\&\quad\left[\textit{IgG and/or F}\left(ab'\right)2\right]\ \textit{in a vial}\,(\text{mg})\end{aligned}$$

The venom-specific antibodies in PAVs from the Indian subcontinent determined from spectrofluorometric titration curve showed varying percentage of venom specific antibodies ranging from 7 to 14% against the venoms of the "Big Four" snakes of India. The results of this analysis were quite similar to those obtained when the venom-specific antibodies of Indian antivenom and antivenom produced against Sri Lankan venoms (raised against *Naja. naja*, *Daboia ruselii*, *Echis carinatus*, and *Hypnale hypnale* from Sri Lanka, produced by Instituto Clodomiro Picado, San José, Costa Rica) against *D. russelii* venom analyzed by third generation antivenomics study.[42] Therefore, both analytical techniques (spectrofluorometric interaction and third generation antivenomics) can be used to determine the venom-specific antibodies of antivenom depending upon the availability of infrastructure and facilities. Figure 3 shows schematic diagrams for the methodologies of determination of venom-specific antibodies by third generation antivenomics and by the spectrofluorometric titration curve of antivenom–venom interaction. Another approach to quantify specific antibodies in an antivenom directed against venom components is by running an ELISA using an antivenom antibody standard purified by affinity chromatography with venom (for details, see Segura et al.[59]).

4.5 | *In vitro* neutralization of enzymatic activities and pharmacological properties of Viperidae venom proteins by antivenoms

Although the pre-clinical assessment to determine the *in vivo* neutralization of lethality and other toxic effects of venoms is the gold

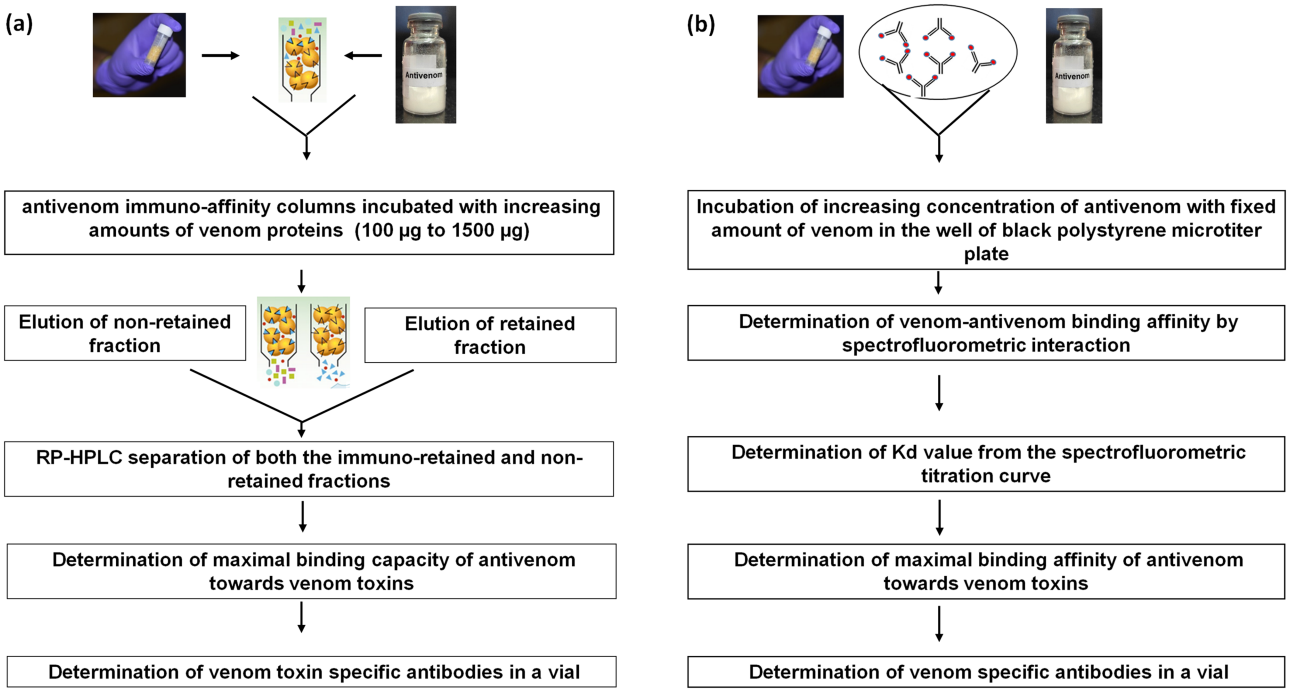

FIGURE 3 Schematic diagram for determination of venom-specific antibodies by (a) third generation antivenomics approach[42,66,128] and (b) one site binding spectrofluorometric titration curve of antivenom–venom interaction[47]

standard to determine the efficacy of antivenom, prior to *in vivo* preclinical assessment, the ability of antivenoms to react with venoms can also be assessed by determining the immunological cross-reactivity by ELISA, Western blot, and antivenomics, as discussed. In addition, the assessment of the neutralization of selected enzymatic activities and pharmacological properties of venoms, particularly those of the Viperidae family, can further expand the platform of *in vitro* tests to evaluate antivenoms.[41,106] Viperidae venoms are rich in enzymatic toxins like PLA_2, snake venom metalloprotease (SVMP), and snake venom serine protease (SVSP), which play an important role in toxicity.[41,110,130–132] Therefore, assessment of *in vitro* neutralization of the enzymatic activities of these venom components is another tool to determine the efficacy of commercial antivenoms. For example, a correlation between the neutralization of PLA_2 activity and the neutralization of lethality has been described for some venoms.[133,134]

Neutralization of enzymatic activities and pharmacological properties of *D. russelli* venom from different geographical locales showed differential cross-reactivity of commercial PAV. For the *in vitro* neutralization of these activities, a fixed quantity of venom is incubated with graded concentrations of antivenom at room temperature for 30 min followed by assay of the corresponding enzyme activity by standard biochemical analysis. The activity of a particular enzyme of venom is considered as 100% activity and the activity of same enzyme in presence of antivenom is compared to that. From the graph, the percent inhibition of enzyme activity by a particular dose of antivenom can be determined.[106,108,113] For example, enzymatic activities and pharmacological properties, except for PLA_2 and indirect hemolytic activities, of *D. russelii* venom from specimens of southern India were neutralized by the commercial antivenoms when compared with the western and eastern India.[41,42,116] These findings correlated with ELISA, Western blot, and antivenomics studies.[41] Therefore, *in vitro* neutralization of snake venom enzymes can be a useful parameter to evaluate the efficacy of antivenoms before their *in vivo* lethality neutralization assessment in rodents.

5 | STABILITY STUDIES OF ANTIVENOMS

Stability testing of pharmaceutical products is necessary to define their ability to maintain their physicochemical, therapeutic, and microbiological properties post storage and use. Many factors can affect the stability of pharmaceutical products, such as storage conditions (e.g., temperature, humidity, and light), dosage form (e.g., particle size, pH, solvents, excipients, and ionic strength), and the primary container.[135] In the case of snake antivenoms, the main degradation pathways of the antibodies are denaturation and aggregation.[136] These instabilities could produce changes in the secondary and tertiary structure of proteins, with loss of the neutralizing activity and a potential increase in immunogenicity and the likelihood of adverse reactions. The WHO, as well as other regional or governmental entities, has issued recommendations and guidelines for carrying out stability studies for biological formulations.[14,137] According to WHO antivenom guidelines,[14] the stability test should be done when a new product is developed, a change in the manufacturing process is introduced, or a new formulation is generated. The frequency of testing, as

well as the storage conditions and the selection of batches, should be decided according to recommendations of the authorities, and based on the manufacturer previous knowledge about the product.

In the case of antivenoms, some of the tests that need to be run in stability studies are *in vivo* venom neutralizing potency, turbidity, aggregate content, appearance, sterility, endotoxin assay, osmolality, pH, total protein, residual humidity, and reconstitution time (the latter two in the case of freeze-dried antivenoms). Real-time stability tests are performed under the expected storage conditions. On the other hand, in accelerated studies, the antivenom is exposed to more extreme conditions than usual, and the stability is assessed over a shorter time.[14] Currently, with the introduction of new tools for protein analysis (e.g., mass spectrometry, differential scanning calorimetry, surface plasmon resonance, and infrared spectroscopy), it is possible to reduce the use of animal models in the quality control assessment of antivenoms.

Overall, considering the high cost of antivenom treatment and the shortage crisis of this essential medicine in the tropical regions of the world, stability issues could have a remarkable effect from a clinical, social, and economic point of view. A rational design and quality control of new antivenoms, as well as the use of available information on the thermal stress of antibodies, could save time and avert problems related to antivenom safety and stability.

6 | CONCLUSIONS AND FUTURE PERSPECTIVES

The WHO global strategy for the prevention and control of snakebite envenomations places particular emphasis in the improvement of the quality and quantity of antivenoms in order to ensure their safety and efficacy and to improve availability and accessibility.[8] The universe of antivenom manufacturers is highly heterogeneous and in need of a systematic effort to implement GMPs and to improve several aspects of the manufacturing technology.[50] Several factors determine the safety and efficacy of antivenoms, such as physicochemical properties, purity of the active (therapeutic) molecule, protein aggregates, endotoxin contamination, and presence of venom/toxin-specific antibodies effective in the neutralization of venom toxins. Failure to comply with GMPs and the use of methodologies that generate substandard products are responsible for the poor efficacy and safety of some antivenoms, an issue that needs to be properly addressed and solve through international cooperation.

The protocols discussed in this communication provide a methodological platform that could be highly useful for laboratories working in the development, manufacture, and quality control of antivenoms, as well as for national regulatory agencies. Some of these procedures are indicated in the routine quality control of antivenoms. Other methods are not aimed at the routine control but constitute valuable tools to evaluate and improve the protocols used in antivenom manufacture in order to generate higher quality antivenoms. An international partnership is required to upgrade antivenom manufacturing facilities through a scheme of cooperation involving laboratories with expertise in the methodologies described. The concerted participation of manufactures, research groups, international health institutions, and national regulatory agencies could promote the use of these laboratory tools for the improvement of antivenom quality on a global basis.

ACKNOWLEDGEMENTS

AP received Senior Research Fellowship from Department of Science and Technology, Ministry of Science and Technology, India, Indo-ASEAN project. This study received grant from DST Indo-ASEAN project (INRC/AISTDF/R&D/P-3/2017) to AKM.

CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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How to cite this article: Patra A, Herrera M, Gutiérrez JM, Mukherjee AK. The application of laboratory-based analytical tools and techniques for the quality assessment and improvement of commercial antivenoms used in the treatment of snakebite envenomation. *Drug Test Anal*. 2021;13:1471–1489. https://doi.org/10.1002/dta.3108