Annex 5

Guidelines for the production, control and regulation of snake antivenom immunoglobulins

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Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA.

Abbreviations

ASV	anti-snake venom
BVDV	bovine viral diarrhoea virus
CK	creatine kinase
CPD	citrate phosphate dextrose solution
CTD	Common Technical Document
ds-DNA	double-stranded deoxyribonucleic acid
ds-RNA	double-stranded ribonucleic acid
ED_{50}	effective dose 50%
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMCV	encephalomyocarditis virus
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GCP	good clinical practice
GMP	good manufacturing practice(s)
Hb	haemoglobin
HPLC	high-performance liquid chromatography
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IgG	immunoglobulin G
IgM	immunoglobulin M
LD_{50}	lethal dose 50%
MCD	minimum coagulant dose
MDD	minimum defibrinogenating dose
MHD	minimum haemorrhagic dose
MHD_{50}	MHD-median effective dose
MMD	minimum myotoxic dose

MMD_{50}	MMD-median effective dose
MND	minimum necrotizing dose
MND_{50}	MND-median effective dose
Mr	relative molecular mass
NRA	national regulatory authority
PCV	packed cell volume
RCT	randomized controlled trial
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SOP	standard operating procedure
ss-DNA	single-stranded deoxyribonucleic acid
ss-RNA	single-stranded ribonucleic acid
TPP	total plasma protein
TSE	transmissible spongiform encephalopathy
VSV	vesicular stomatitis virus
WNV	West Nile virus

1. Introduction

Snake antivenom immunoglobulins (antivenoms) are the only therapeutic products for the treatment of snake-bite envenoming. The lack of availability of effective snake antivenom immunoglobulins to treat envenoming by medically important venomous snakes encountered in various regions of the world has become a critical health issue at global level. The crisis has reached its greatest intensity in sub-Saharan Africa, but other regions, such as South and South-East Asia, are also suffering from a lack of effective and affordable products.

The complexity of the production of efficient antivenoms, in particular the importance of preparing appropriate snake venom mixtures for the production of hyperimmune plasma (the source of antivenom immunoglobulins), the decreasing number of producers, and the fragility of the production systems in developing countries further jeopardize the availability of effective antivenoms in Africa, Asia, the Middle East and South America. Most of the remaining current producers are located in countries where the application of quality and safety standards needs to be improved.

In October 2005, the WHO Expert Committee on Biological Standardization recognized the extent of the problem and asked the WHO Secretariat to support and strengthen world capacity to ensure the long-term and sufficient supply of safe and efficient antivenoms. In March 2007, snake antivenom immunoglobulins were included in the WHO Model List of Essential Medicines (*1*), acknowledging their role in a primary health-care system.

WHO recognizes that urgent measures are needed to support the design of immunizing snake venom mixtures that can be used to make appropriate antivenoms for various geographical areas of the world. Sustainable availability of effective and safe antivenom immunoglobulins must be ensured and production systems for these effective treatments must be strengthened at global level. Meaningful preclinical assessment of the neutralizing capacity of snake antivenom immunoglobulins needs to be done before these products are used in humans and medicines regulatory authorities should enforce the licensing of these products in all countries, before they are used in the population.

The first edition of the WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins was developed in response to the above-mentioned needs and approved by the WHO Expert Committee on Biological Standardization in October 2008. These Guidelines covered all the steps involved in the production, control and regulation of venoms and antivenoms. The Guidelines are supported by a WHO antivenoms database website[1] that

[1] See: http://apps.who.int/bloodproducts/snakeantivenoms/database/ (accessed 15 February 2017).

features information on all the venomous snakes listed in Appendix 1, including distributions and photographs, as well as information on available antivenoms.

It is intended that these updated Guidelines, by comprehensively describing the current existing experience in the manufacture, preclinical and clinical assessment of these products, will serve as a guide to national regulatory authorities (NRAs) and manufacturers in support of worldwide production of these essential medicines. The production of snake antivenoms following good manufacturing practices (GMP) should be the aim of all countries involved in the manufacture of these life-saving biological products.

In addition to the need to produce appropriate antivenoms, there is a need to ensure that antivenoms are appropriately used and that outcomes for envenomed patients are improved. This entails improving availability and access to antivenoms, appropriate distribution policies, antivenom affordability, and training of health workers to allow safe, selective and effective use of antivenoms and effective management of snake-bite envenoming. These important issues are beyond the scope of this document and will not be further addressed specifically here, but should be considered as vital components in the care pathway for envenoming.

This second edition of the Guidelines was prepared in 2016 in order to ensure that the information contained in these sections remains relevant to the current production of snake antivenom immunoglobulins and their subsequent control and regulation.

Major updates in this second edition include:

- inclusion of stronger animal welfare and ethical compliance messages (section 4) to reinforce the importance of humane use of animals in the production of antivenoms;
- updates to lists of medically important snakes to reflect new species discoveries and recent nomenclatural changes (section 7; and Appendix 1);
- revision of methodologies for serpentariums that produce venoms to emphasize traceability and quality control, including the recommendation to discontinue use of wild-capture/release strategies for ethical and quality control reasons (section 9);
- increased emphasis on the specific health control of plasma donor animals, particularly prior to, and during plasma collection session (sections 12 and 14);
- updated lists of known potential equine virus contaminants (section 16);

- redrafting and reorganization of sections on the quality control (section 17), stability studies (section 18) and preclinical assessment (section 19) of antivenoms, to incorporate new approaches and technologies, and eliminate repetition;
- revised information on the clinical assessment of antivenoms (section 20), as well as an expanded and strengthened discussion on the role of NRAs and the need for national reference venom collections independent from antivenom manufacturers (section 21; see also section 10.3).

2. Purpose and scope

These WHO Guidelines provide guidance to NRAs and manufacturers on the production, control and regulation of snake antivenom immunoglobulins. It should however be recognized that some sections, such as: those dealing with immunogen quality control, reference materials, and the production, purification and testing of antibodies (sections 10–19); as well as most of the guidance which deals with regulatory oversight (section 21); and the ethical use of laboratory animals and plasma donor animals (section 4); may also apply to other types of antivenoms, such as those produced for the treatment of envenoming caused by spiders, scorpions and other organisms. There are also other immunoglobulin products of animal origin for which some of the production methodologies described here may be similar or identical – for example, the selection and veterinary health care of animals; immunization regimens and use of adjuvants; collection and control of animal plasma for fractionation; purification of immunoglobulins; and control of infectious risks. These WHO Guidelines may therefore have application beyond providing information for the production of snake antivenom immunoglobulins, and may be applicable also to other antivenoms or animal-derived immunoglobulin products (for example, equine-derived botulism antitoxins).

3. Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

Antivenom – also called antivenin or anti-snake venom (ASV): a purified fraction of immunoglobulins or immunoglobulin fragments fractionated from the plasma of animals that have been immunized against one or more snake venoms.

Apheresis: procedure whereby blood is removed from the donor, separated by physical means into components and one or more of them returned to the donor.

Batch: a defined quantity of starting material or product manufactured in a single process or series of processes so that it is expected to be homogeneous.

Batch records: all documents associated with the manufacture of a batch of bulk product or finished product. They provide a history of each batch of product and of all circumstances pertinent to the quality of the final product.

Blood collection: a procedure whereby a single donation of blood is collected in an anticoagulant and/or stabilizing solution, under conditions designed to minimize microbiological contamination of the resulting donation.

Bulk product: any product that has completed all processing stages up to, but not including, aseptic filling and final packaging.

Clean area: an area with defined environmental control of particulate and microbial contamination constructed and used in such a way as to reduce the introduction, generation, and retention of contaminants within the area.

Contamination: the undesired introduction of impurities of a microbiological or chemical nature, or of foreign matter, into or on to a starting material or intermediate during production, sampling, packaging, or repackaging, storage or transport.

Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES): an international agreement between governments that aims to ensure that international trade in specimens of wild animals and plants does not threaten their survival.

Cross-contamination: contamination of a starting material, intermediate product or finished product with another starting material or product during production.

Cross-neutralization: the ability of an antivenom raised against a venom, or a group of venoms, to react and neutralize the toxic effects of the venom of a related species not included in the immunizing venom mixture.

Common Technical Document (CTD) format: a specific format for product dossier preparation recommended by WHO and the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

Desiccation: a storage process where venoms are dehydrated under vacuum in the presence of calcium salts or phosphoric acid.

Effectiveness: the effectiveness of an antivenom is a measure of its ability to produce a clinically effective outcome when used to treat snake-bite envenoming.

Efficacy: the efficacy of an antivenom is a measure of the in vivo or in vitro neutralizing potency against a specific activity of a venom or venoms.

Envenoming: injection of venom by an organism (for example, venomous snake) into another organism, leading to pathological manifestations (also called envenomation).

Fab: an antigen-binding fragment (Fab) of an immunoglobulin comprising a heavy chain and a light chain that each have a single constant domain and a single variable domain. Fab fragments result from the proteolytic digestion of immunoglobulins by papain (or pepsin after $F(ab')_2$ digestion).

$F(ab')_2$: an immunoglobulin fragment comprising a pair of Fab fragments connected by a protein hinge, and produced by proteolytic digestion of whole immunoglobulins with pepsin.

Fractionation: large-scale process by which animal plasma is separated to isolate the immunoglobulin fraction that is further processed for therapeutic use or may be subjected to digestion with pepsin or papain to generate immunoglobulin fragments. The term fractionation is generally used to describe a sequence of processes, usually including plasma protein precipitation and/or chromatography, ultrafiltration and filtration steps.

Freund's complete adjuvant (FCA): an adjuvant that may be used in the immunization process of animals to enhance the immune response to venoms. It is composed of mineral oil, an emulsifier and inactivated *Mycobacterium tuberculosis*.

Freund's incomplete adjuvant (FIA): an adjuvant that may be used in the immunization process of animals to enhance the immune response to venoms. It is composed of mineral oil and an emulsifier.

Good clinical practice (GCP): an international standard for rigorous, ethical and high quality conduct in clinical research, particularly in relation to all aspects of the design, conduct, analysis, record-keeping, auditing and reporting of clinical trials involving human subjects. GCP standards are established by the ICH under Topic E 6 (R1).

Good manufacturing practice (GMP): that part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization or product specification. It is concerned with both production and quality control.

Immunization process: a process by which an animal is injected with venom(s) to produce a high-titre antibody response against the lethal and other deleterious components in the immunogen.

Immunoglobulin: immune system forming protein produced by B-cells in plasma that can recognize specific antigens. These can be generated by immunizing an animal (most often a horse) against a snake venom or a snake venom mixture. Immunoglobulin G (IgG) is the most abundant immunoglobulin fraction.

Immunoglobulin G (IgG): one of the five classes of antibodies produced by the B-cells. It is synthesized in response to invasions by bacteria, fungi and viruses. IgG crosses the placenta and protects the fetus. It is a complex protein composed of four peptide chains – two identical heavy chains and two identical light chains arranged in a typical Y shape of antibody monomers. Representing approximately 75% of serum antibodies in humans, IgG has a molecular mass of approximately 150 kDa.

Immunoglobulin M (IgM): another type of antibody. It is an immunoglobulin of high molecular weight that is released into the blood early in the immune response to be replaced later by IgG and is highly efficient in binding complement. IgM antibodies make up about 5 to 10% of all the antibodies in the body; they have a polymeric form, mostly as pentamers. IgM has a molecular mass of approximately 970 kDa.

In-process control: checks performed during production to monitor and, if necessary, to adjust the process to ensure that the antivenom conforms to specifications. The control of the environment or equipment may also be regarded as part of in-process control.

Manufacture: all operations of purchase of materials and products, production, quality control, release, storage and distribution of snake antivenom immunoglobulins, and the related controls.

Median effective dose – or effective dose 50% (ED_{50}): the quantity of antivenom that protects 50% of test animals injected with a median lethal dose of venom.

Median lethal dose – or lethal dose 50% (LD_{50}): the quantity of snake venoms, injected intravenously or intraperitoneally, that leads to the death of 50% of the animals in a group after an established period of time (usually 24–48 hours).

Minimum coagulant dose (MCD): the minimum amount of venom (in mg/L or µg/mL) that clots either a solution of bovine fibrinogen (2.0 g/L) in 60 seconds at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (2.8 g/L fibrinogen) under the same conditions (MCD-P).

Minimum coagulant dose-F-effective dose ($MCD\text{-}F_{100}$) and MCD-P-effective dose ($MCD\text{-}P_{100}$): the minimum volume of antivenom or venom/antivenom ratio, which completely prevents clotting induced by either one MCD-F or MCD-P dose of venom.

Minimum defibrinogenating dose (MDD): the minimum amount of venom that produces incoagulable blood in all mice tested within one hour of intravenous injection.

Minimum defibrinogenating dose-effective dose (MDD_{100}): the minimum volume of antivenom or venom/antivenom ratio, at which the blood

samples of all injected mice show clot formation after administration of one or more MDD doses of venom.

Minimum haemorrhagic dose (MHD): The minimum amount of venom (in μg) that when injected intradermally in mice causes a 10 mm haemorrhagic lesion within a predefined time interval (for example, 2–3 hours).

Minimum haemorrhagic dose-median effective dose (MHD_{50}): the minimum volume of antivenom (in μL) that reduces the diameter of haemorrhagic lesions by 50% compared to those induced in animals who receive a control solution of venom/saline.

Minimum myotoxic dose (MMD): the minimum amount of venom that produces a four-fold increase in serum or plasma creatine kinase (CK) activity above that of control animals.

Minimum myotoxic dose-median effective dose (MMD_{50}): the minimum amount of antivenom (in μL or the venom/antivenom ratio) that reduces the serum or plasma CK activity by 50% compared to those induced in animals who receive a control solution of venom/saline.

Minimum necrotizing dose (MND): the minimum amount of venom (in μg) that when injected intradermally in groups of lightly anaesthetized mice results in a necrotic lesion 5 mm in diameter within 72 hours.

Minimum necrotizing dose-median effective dose (MND_{50}): the minimum amount of antivenom (in μL or the venom/antivenom ratio) that reduces the diameter of necrotic lesions by 50% compared to those induced in animals who receive a control solution of venom/saline.

Monospecific antivenom: antivenoms that are raised from venom of a single species, and are limited in use to that species or to a few closely related species (typically from the same genus) whose venoms show clinically effective cross-neutralization with the antivenom. The term "monovalent" is often used and has the same meaning.

Nanofilter: filters, most typically with effective pore sizes of 50 nm or below, designed to remove viruses from protein solutions.

National regulatory authority (NRA): WHO terminology to refer to national medicines regulatory authorities. Such authorities promulgate medicine regulations and enforce them.

Plasma: the liquid portion remaining after separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure.

Plasmapheresis: procedure in which whole blood is removed from the donor, the plasma is separated from the cellular elements by sedimentation, filtration, or centrifugation, and at least the red blood cells are returned to the donor.

Polyspecific antivenom: antivenoms that are obtained by fractionating the plasma from animals immunized with a mixture of venoms from more than one species of venomous snake. The term "polyvalent" is often used and has the same meaning.

Prion: a particle of protein that is thought to be able to self-replicate and to be the agent of infection in a variety of diseases of the nervous system, such as scrapie, mad cow disease and other transmissible spongiform encephalopathies (TSEs). It is generally believed not to contain nucleic acid.

Production: all operations involved in the preparation of snake antivenom immunoglobulins, from preparation of venoms, immunization of animals, collection of blood or plasma, processing, packaging and labelling, to its completion as a finished product.

Quality manual: an authorized, written controlled document that defines and describes the quality system, the scope and operations of the quality system throughout all levels of production, management responsibilities, key quality systems processes and safeguards.

Quarantine: a period of enforced isolation and observation typically to contain the spread of an infectious disease among animals. The same terminology applies to the period of isolation used to perform quality control of plasma prior to fractionation, or of antivenom immunoglobulins prior to release and distribution.

Randomized controlled trial (RCT): randomized controlled trial of a pharmaceutical substance or medical device.

Serpentarium: a place where snakes are kept, for example, for exhibition and/or for collection of venoms.

Serum: a liquid portion remaining after clotting of the blood. Serum has a composition similar to plasma (including the immunoglobulins) apart from fibrinogen and other coagulation factors which constitute the fibrin clot.

Site Master File: an authorized, written controlled document containing specific factual details of the GMP production and quality control manufacturing activities that are undertaken at every site of operations linked to products that a company produces.

Standard operating procedure (SOP): an authorized written procedure giving instructions for performing operations not necessarily specific to a given product or material (for example, equipment operation, maintenance and cleaning; validation; cleaning of premises and environmental control; sampling and inspection). Certain SOPs may be used to supplement product-specific master and batch production documentation.

Toxin: a toxic substance, especially a peptide or protein, which is produced by living cells or organisms and is capable of causing disease when introduced into the body tissues. It is often also capable of inducing neutralizing antibodies or antitoxins.

Traceability: ability to trace each individual snake, venom, immunized animal, or unit of blood or plasma used in the production of an antivenom immunoglobulin with each batch of the final product. The term is used to describe forward and reverse tracing.

Validation: action of proving, in accordance with the principles of GMP, that any procedure, process, equipment, material, activity, or system actually leads to the expected results.

Venom: the toxic secretion of a specialized venom gland which, in the case of snakes, is delivered through the fangs and provokes deleterious effects. Venoms usually comprise many different protein components of variable structure and toxicity.

Venom extraction – or venom collection or "milking": The process of collecting venom from live snakes.

Viral inactivation: a process of enhancing viral safety in which viruses are intentionally "killed".

Viral reduction: a process of enhancing viral safety in which viruses are inactivated and/or removed.

Viral removal: a process of enhancing viral safety by partitioning viruses from the components of interest.

4. The ethical use of animals

Current methods of antivenom production rely on the use of animals to manufacture these life-saving products. For all animals, whether they are venomous snakes from which venom is obtained for use as an immunogen; the horses, sheep or other large animals that are injected with the venom, and serve as living antibody factories, producing hyperimmune plasma from which antivenom is derived; or the small laboratory animals sacrificed in order to test the preclinical efficacy and safety of antivenoms, there is an absolute necessity for all manufacturers to use animals humanely and ethically.

It is imperative that venom producers, antivenom manufacturers and quality control laboratories that make use of animals in venom or antivenom research, production, or in the preclinical evaluation of antivenoms adhere to the highest ethical standards. The *International guiding principles for biomedical research involving animals* (2012) developed by the International Council for Laboratory Animal Science and the Council for International Organization of Medical Sciences provide an international benchmark for the use of animals in research. Compliance with national guidelines, laws and regulations is also essential. All animal experimentation should be subject to regulatory oversight at an institutional and national level. In many jurisdictions, the 3R principles of Replacement, Reduction and Refinement have been adopted as cornerstones

of ethical use of animals, and WHO strongly recommends that every effort be made to reduce pain, distress and discomfort to experimental animals – for example, by the routine use of analgesia in mice used in these assays.

4.1 Ethical considerations for the use of venomous snakes in the production of snake venoms

Venomous snakes kept in serpentariums for use in venom production should be maintained according to nationally and internationally accepted ethical standards. All relevant local regulations should be strictly adhered to, and where required the use of venomous snakes in venom production should be conducted in accordance with ethics approvals obtained from responsible authorities in the jurisdiction. This particularly applies to the collection of wild specimens and their transportation to serpentariums. It is important that specimens be sourced from legal suppliers, and venom producers should ensure that the collection localities of all specimens are known, and that evidence of legal collection is supplied. As discussed in section 9.1.4.2 the practice of capturing wild venomous snakes, extracting venom and releasing the snakes after translocation into new habitat must be discontinued. This is not just because of issues relating to traceability and quality control, which are fundamental to production of antivenoms in accordance with GMP, but also because mounting evidence demonstrates unacceptably high mortality among translocated venomous snakes. Compliance with local ethical requirements for the keeping of venomous snakes in captivity, the humane handling of specimens, veterinary care and supervision, and euthanasia (when necessary for humane reasons) should be maintained. Another important consideration for serpentariums is the necessity to use other animals as food sources for venomous snakes. The types of animals used as food, their production, humane euthanasia, or in some cases, presentation to snakes as live prey, require appropriate ethical considerations, and specific licences and ethics approvals may be required to keep, breed and use some animals as sources of food for venomous snakes. Venom producers must ensure that their operations comply with all necessary regulations and requirements in this regard.

4.2 Ethical considerations for the use of large animals in the production of hyperimmune plasma

The use of large animals (for example, horses, ponies, mules and sheep) in the production of hyperimmune plasma requires constant veterinary supervision and strict adherence to approved national and international ethical standards for these animals. Equines are the most commonly used for production of

hyperimmune plasma in antivenom production and have specific physiological and psychological requirements for good health and the minimization of pain and distress. Manufacturers must recognize these needs and structure their use of animals to ensure that their social, physical and environmental needs are appropriately met. Relevant guidelines and regulations established by competent authorities should be implemented. Veterinary care of animals should meet the highest standards, and the health and welfare of individual animals used for plasma production should be closely monitored at all times. The process of immunizing donor animals with snake venoms raises important ethical considerations, particularly because of the potential harm that can be caused by some venoms (for example, neurotoxins, necrotic or cytotoxic venoms) and by the adjuvants that are used in most immunization protocols, particularly Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA). Animals used in plasma production may suffer considerable distress, pain or discomfort as a result of the immunization process and all manufacturers have an obligation to strictly comply with animal welfare and ethical use requirements and actively work to minimize these deleterious effects. Similarly, the bleeding of animals to collect hyperimmune plasma can be traumatic for donor animals if appropriate techniques are not used to minimize negative effects, including fear, pain, distress and physical harm. Manufacturers are encouraged very strongly to proactively improve the welfare of large animals used in plasma production, and to develop protocols that reduce suffering and improve the health of animals.

4.3 Ethical considerations for the use of animals in preclinical testing of antivenoms

The preclinical testing of new or existing antivenoms necessitates the use of experimental animals, typically rodents, particularly for essential median lethal venom dose (LD_{50}) and median effective antivenom dose (ED_{50}) determination. Despite reservations about the physiological relevance of these animal models to human envenoming and the harm that these in vivo assays cause to the animals (sections 19.2 and 19.3), they are used by both manufacturers and regulatory authorities worldwide for determining venom lethality (LD_{50}) and antivenom neutralizing capacity (ED_{50}) as these are currently the only validated means of assessing venom toxicity and antivenom neutralizing potency. Non-sentient or in vitro assays as alternatives to the standard venom LD_{50} and antivenom ED_{50} in vivo tests have been promoted (*2*). Unfortunately, such systems have not been developed to the point where they can fully replace the above-mentioned preclinical assays. In the absence of effective alternatives, the continued use of experimental animals is still justified by the considerable benefits to human health of these preclinical assays.

4.4 Development of alternative assays to replace murine lethality testing

In vivo murine assays cause considerable suffering and a 3R approach involving innovation and validation should be applied in the development of standardized LD_{50} and ED_{50} test protocols. Designing protocols that use the minimal number of animals necessary and introducing procedures to minimize pain and suffering is essential. The development of alternative methods to replace animal testing in the preclinical evaluation of antivenoms should be encouraged. When tests on live animals are absolutely necessary, anaesthesia or analgesia should be considered and evaluated to ensure that the humane benefits of these interventions to the experimental animals do not invalidate the objectives of the assay by altering relevant physiological processes (*3*). In particular, the use of analgesia is recommended when working with venoms that induce tissue damage, and experimental evidence demonstrates convincingly that opioid drugs relieve suffering without altering critical end-points such as LD_{50} and ED_{50} (*4*). The establishment of humane end-points to reduce suffering and limiting the duration of the assays to reduce the period of animal suffering is encouraged; this requires appropriate standardization and validation within a quality assurance framework.

4.5 Refinement of the preclinical assay protocols to reduce pain, harm and distress to experimental animals

The substantial suffering caused to small animals by the preclinical assays is outweighed by the considerable benefits to human health. Nevertheless, WHO strongly encourages that opportunities to implement alternatives to the essential and supplementary tests, according to the 3R, to reduce pain, harm and distress be tested. Thus, designing protocols that use the minimum number of animals necessary and introducing procedures to minimize pain and suffering is essential. Analgesia should be considered, and evaluated to ensure that the humane benefits of analgesia to the experimental animals do not invalidate the objectives of the assay by altering relevant physiological processes (*3*). In particular, the use of analgesia is recommended when working with venoms that induce tissue damage (*4*). The establishment of humane end-points, instead of using survival/death as the assay metric, is encouraged to reduce suffering and limit the duration of the assays. The use of humane end-points also offers the opportunity to introduce 'dose-staging' into the experimental design (in which multiple doses are prepared for the assays, one dose given and the next dose(s) selected based on the results of giving the previous dose) to reduce the number of mice required for these assays. All such efforts towards 3R require appropriate standardization and validation within a quality assurance framework.

4.6 Main recommendations

- **It is imperative that venom producers, antivenom manufacturers and quality control laboratories that use animals in venom or antivenom production, in research or in the preclinical evaluation of antivenoms adhere to the highest ethical standards.**
- **Relevant national and international animal welfare and ethical use guidelines and regulations should be adhered to.**
- **Wherever possible, alternative protocols and procedures that minimize pain, suffering and physical or psychological distress to animals should be developed and validated.**
- **The 3R approach should be applied in the development of standardized and validated LD_{50} and ED_{50} test protocols.**

5. General considerations

Snake antivenom immunoglobulins – antivenoms, antivenins, anti-snake-bite serum and anti-snake venom (ASV) – are the only specific treatment for envenoming by snake-bites. They are produced by the fractionation of plasma that is usually obtained from large domestic animals hyperimmunized against relevant venoms. Important but seldom used antivenoms may be prepared in smaller animals. In general, when injected into an envenomed human patient, an effective antivenom will neutralize toxins in any of the venoms used in its production, and in some instances, will also neutralize venoms from closely related species.

5.1 Historical background

Shortly after the identification of diphtheria and tetanus toxins, von Behring and Kitasato reported the antitoxic properties of the serum of animals immunized against diphtheria or tetanus toxins and suggested the use of antisera for the treatment of these diseases (*5*). In 1894, von Behring diphtheria antitoxin was first successfully administered by Roux to save children suffering from severe diphtheria. Thus, serum therapy was born and the antitoxin was manufactured by Burroughs Wellcome in the United Kingdom. The same year, Phisalix and Bertrand (*6*) and Calmette (*7*) simultaneously, but independently, presented during the same session of the same meeting their observations on the antitoxic properties of the serum of rabbits and guinea-pigs immunized against cobra and viper venoms, respectively. Immediately after his discovery of "antivenin serum therapy", Calmette became actively involved in proving its effectiveness in the

treatment of human envenoming. The first horse-derived antivenom sera that he prepared were in clinical use in 1895 by Haffkine in India and by Lépinay in Viet Nam. The latter reported the first successful use of antivenin serum therapy in patients in 1896 (*8*).

5.2 The use of serum versus plasma as source material

Historically, the pioneers, Calmette, Vital Brazil and others, used serum separated from the blood of hyperimmunized horses for the preparation of antivenom ("antivenin serum therapy"). Later, antibodies (immunoglobulins) were demonstrated to be the active molecules responsible for the therapeutic action of "antivenom serum". Subsequently, immunoglobulins, or immunoglobulin fragments ($F(ab')_2$, Fab), purified from serum were used instead of crude serum (*9*, *10*). Nowadays, plasmapheresis, whereby red blood cells are re-injected into the donor animal within 24 hours of blood collection, is commonly employed to reduce anaemia in the hyperimmunized animal that donates the plasma. Accordingly, it is almost exclusively plasma rather than serum, which is used as the starting material for the extraction of the immunoglobulin or its fragments (*11*–*13*). Thus "snake antivenom immunoglobulin" is the preferred term, rather than "anti-snake-bite serum" or "antiserum" which are no longer accurate.

5.3 Antivenom purification methods and product safety

The recognition of their role, and the purification of immunoglobulins from other components of the serum or plasma of donor animals, was pioneered in the earliest years of the last century using simple chemical reactions (*14*–*18*). The subsequent discovery, more than half a century later, of the structure of antibodies opened new doors to the fractionation of immunoglobulins. It became possible to produce antibody fractions ($F(ab')_2$ or Fab) that were believed to potentially reduce the frequency of early and late antivenom reactions by removing the Fc fragment from IgG (*19*). This was subsequently believed to prevent complement activation and perhaps reduce the intensity of immune-complex formation responsible for late antivenom reactions (serum sickness). For 60–70 years, immunoglobulin $F(ab')_2$ fragments have been widely used. However, antivenom protein aggregation, and not Fc-mediated complement activation, was increasingly identified as a major cause of antivenom reactions. Thus, a critical issue in antivenom safety probably lies in the physicochemical characteristics of antivenoms and not exclusively in the type of neutralizing molecules constituting the active substance. It is also important to ensure that the current methods of producing antivenoms provide a sufficient margin of safety with regard to the potential risk of transmission of zoonoses.

5.4 Pharmacokinetics and pharmacodynamics of antivenoms

Rapid elimination of some therapeutic antivenoms (for example, when Fab fragments are used) has led to recurrence of envenoming in patients. However, the choice of preparing specific IgG or fragments appears to depend on the size and toxicokinetics of the principal toxin(s) of the venoms. Large relative molecular mass (Mr) bivalent antibodies (IgG and $F(ab')_2$ fragments) may be effective for the complete and prolonged neutralization of intravascular toxins (for example, procoagulant enzymes), which have a long half-life in envenomed patients. Low Mr and monovalent IgG fragments, such as Fab, may be more appropriate against low-molecular-mass neurotoxins which are rapidly distributed to their tissue targets and are rapidly eliminated from the patient's body, for example, scorpion and spider toxins (*20*).

5.5 Need for national and regional reference venom preparations

Antivenom production is technically demanding. The need to design appropriate monospecific or polyspecific antivenoms (depending on the composition of the snake fauna) is supported by the difference in venom composition among venomous animals, in particular bearing in mind that:

- many countries can be inhabited by several medically important species;
- there may be wide variation in venom composition (and hence antigenicity) through the geographical range of a single species;
- in some circumstances there is no distinctive clinical syndrome to direct the use of monospecific antivenoms.

However, similarities in the venom toxins of closely related venomous species may result in cross-neutralization (paraspecific neutralization), thus reducing the number of venoms required for the preparation of polyspecific antivenoms. Cross-neutralization should be tested in animal models and ideally by clinical studies in envenomed patients. Preclinical testing of antivenoms against medically important venoms present in each geographical region or country is a prerequisite for product licences and batch approval, and should always precede clinical use in envenomed patients. This requires efforts by manufacturers and/or regulators to establish regional or national reference venom preparations that can be used to test the neutralization capacity of antivenoms. The national control laboratory of the country where the antivenom will be used, or the manufacturer seeking a licence for the antivenom, should perform such preclinical testing using reference venom preparations relevant to the country or the geographical area.

6. Epidemiological background

The incidence of medically important snake-bites in different parts of the world and the recognition of the species of greatest medical importance is fundamental to the appropriate design of monospecific and polyspecific antivenoms in countries and regions. Up-to-date epidemiological and herpetological information is therefore highly relevant to antivenom manufacturers and regulators, especially for the selection of the most appropriate venoms or venom mixtures to be used in the production and quality control of antivenoms.

6.1 Global burden of snake-bites

Envenoming and deaths resulting from snake-bites are a particularly important public health problem in rural tropical areas of Africa, Asia, Latin America and Papua New Guinea (*21*). Agricultural workers and children are the most affected groups. Epidemiological assessment of the true incidence of global mortality and morbidity from snake-bite envenoming has been hindered by several well recognized problems (*22*, *23*). Snake-bite envenoming and associated mortality are underreported because many victims (20–70% in some studies) do not seek treatment in government dispensaries or hospitals and hence are not recorded. This is compounded by the fact that medical posts in regions of high incidence are unable to keep accurate records of the patients who do present for treatment, and because death certification of snake-bite is often imprecise (*24*, *25*).

Correctly designed population surveys, in which questionnaires are distributed to randomly selected households in demographically well-defined areas, are the only reliable method for estimating the true burden of snake-bites in rural areas. The results of the few such surveys that have been performed have shown surprisingly high rates of bites, deaths and permanent sequelae of envenoming (*25–29*). However, because of the heterogeneity of snake-bite incidence within countries, the results of surveys of local areas cannot be extrapolated to give total national values. Most of the available data suffer from these deficiencies and, in general, should be regarded as underestimates and approximations.

However, the true burden of national snake-bite morbidity and mortality in three South Asian countries has recently been revealed by the results of three well-designed community-based studies. In India, a direct estimate of 46 000 snake-bite deaths in 2005 was derived from the Million Death Study (*30*), in Bangladesh there were an estimated 589 919 snake-bites resulting in 6,041 deaths in 2009 (*31*), and in Sri Lanka in 2012–2013, 80 000 bites, 30 000 envenomings and 400 deaths in one year (*32*). Published estimates of global burden, employing highly controversial methodologies, suggest a range from a minimum of 421 000

envenomings and 20 000 deaths up to as many as 2.5 million cases and more than 100 000 deaths each year (*23*, *33*). In view of the recent data from South Asia, these figures would seem to be underestimates. In addition, the number of people left with permanent sequelae as a result of envenoming is likely to be higher than the number of fatalities (*21*). As already identified, most of the estimated burden of snake-bite is in sub-Saharan Africa, Central and South America and South and South-East Asia.

The current literature on snake-bite epidemiology highlights the inadequacy of the available data on this neglected tropical disease. There is clearly a need to improve reporting and record-keeping of venomous bites in health facilities, to support high-quality epidemiological studies of snake-bite in different regions, and to improve the training of medical personnel. Wherever possible, recording the species that caused the bite as well as death or injury would greatly assist in documenting which species are of clinical significance in individual countries. Making venomous bites notifiable and fully implementing the use of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (*34*) in official death certification (for example, T 63.0 snake venom) would further help to determine the burden of snake-bite more accurately.

6.2 Main recommendations

- **In most parts of the world, snake-bites are underreported and in some parts are completely unreported. This deficiency in surveillance and the paucity of properly designed epidemiological studies explain why the impact of this important public health problem has remained for so long unrecognized and neglected.**
- **National health authorities should be encouraged to improve the scope and precision of their epidemiological surveillance of this disease by:**
  - **improving the training of all medical personnel so that they are more aware of the local causes, manifestations and treatment of venomous bites;**
  - **making venomous bites notifiable;**
  - **setting up standardized and consistent epidemiological surveys;**
  - **improving the reporting and record-keeping of venomous bites by hospitals, clinics, dispensaries and primary health-care posts, and relating the bites to the species of venomous snake that caused the bite wherever possible; and**

– **fully implementing the use of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (2007) (*22*) in official death certification (for example, T 63.0 snake venom).**[2]

7. Worldwide distribution of venomous snakes

7.1 Taxonomy of venomous snakes

Recognizing the species causing the greatest public health burden, designing and manufacturing antivenoms and optimizing patient treatment are all critically dependent on a correct understanding of the taxonomy of venomous snakes. Like other sciences, the field of taxonomy is constantly developing. New species are still being discovered, and many species formerly recognized as being widespread have been found to comprise multiple separate species as scientists obtain better information, often with new technologies. As the understanding of the relationships between species is still developing, the classification of species into genera is also subject to change. The names of venomous species used in these guidelines conform to the taxonomic nomenclature that was current at the time of publication. Some groups of venomous snakes remain under-studied and poorly known. In these cases, the classification best supported by what evidence exists is presented with the limitation that new studies may result in changes to the nomenclature.

Clinicians, toxinologists, venom producers and antivenom manufacturers should endeavour to remain abreast of these nomenclatural changes. These changes often reflect improved knowledge of the heterogeneity of snake populations, and may have implications for venom producers, researchers and antivenom manufacturers. Although taxonomic changes do not necessarily indicate the presence of "new" venoms, they strongly suggest that toxinological and epidemiological research into these "new" taxa may be required to establish their medical relevance, if any.

Since some of the names of medically important species have changed in recent years, the following points are intended to enable readers to relate the current nomenclature to information in the older literature:

- The large group of Asian arboreal pit vipers, which in recent years had been split from a single genus (*Trimeresurus*), into a number of new genera (for example, *Cryptelytrops*, *Parias*, *Peltopelor*, *Himalayophis*, *Popeia*, *Viridovipera*, *Ovophis* and *Protobothrops*, with a few species retained in *Trimeresurus*) based on prevailing

[2] http://www.who.int/classifications/apps/icd/icd10online/ (accessed 15 February 2017).

views of the interrelationships between these groups, have now largely been returned to *Trimeresurus*. There are divergent views on this approach to the taxonomy of these snakes, and interested parties should consult the literature. Some changes made in the early 1980s have gained acceptance and been retained (that is, *Protobothrops*). Medically important species formerly classified in *Cryptelytrops* include *Trimeresurus albolabris*, *T. erythrurus* and *T. insularis*. *Viridovipera stejnegeri* has been returned to *Trimeresurus*.

- It is likely that new species of cobra (*Naja* spp.) will be identified within existing taxa in both Africa and Asia. Three new species (*N. ashei*, *N. mandalayensis* and *N. nubiae*) have been described and several subspecies elevated to specific status since 2000 (for example, *N. annulifera* and *N. anchietae*, from being subspecies of *N. haje*), in addition to the synonymization of the genera *Boulengerina* and *Paranaja* within the *Naja* genus. Such changes may hold significance for antivenom manufacturers and should stimulate further research to test whether existing antivenoms cover all target snake populations.
- Several medically important vipers have been reclassified: *Daboia siamensis* has been recognized as a separate species from *Daboia russelii*; *Macrovipera mauritanica* and *M. deserti* have been transferred to *Daboia*; the Central American rattlesnakes, formerly classified with *Crotalus durissus*, are now *Crotalus simus*; and *Bothrops neuwiedi* has been found to consist of a number of different species, three of which (*B. neuwiedi*, *B. diporus* and *B. mattogrossensis*) may be of public health importance.

It is recognized that there have been many accepted revisions of taxonomy over the past few decades. These WHO Guidelines are aimed at a very wide range of readers, and to assist in matching some old and familiar names with the current nomenclature, Tables A5.1 and A5.2 summarize the major changes made between 1999 and 2016. A list of relevant herpetological references is provided at the end of Appendix 1 of these Guidelines.

Table A5.1
Genus-level name changes (1999–2016)

Currently accepted name	Previous name(s)
Bothrocophias hyoprora	*Bothrops hyoprora*
Bothrocophias microphthalmus	*Bothrops microphthalmus*
Trimeresurus albolabris	*Cryptelytrops albolabris*

Table A5.1 *continued*

Currently accepted name	Previous name(s)
Trimeresurus erythrurus	*Cryptelytrops erythrurus*
Trimeresurus insularis	*Cryptelytrops insularis*, *Trimeresurus albolabris insularis*
Trimeresurus macrops	*Cryptelytrops macrops*
Trimeresurus purpureomaculatus	*Cryptelytrops purpureomaculatus*
Trimeresurus septentrionalis	*Cryptelytrops septentrionalis*, *Trimeresurus albolabris septentrionalis*
Daboia deserti	*Macrovipera deserti*, *Vipera mauritanica deserti*, *Vipera lebetina deserti*
Daboia mauritanica	*Macrovipera mauritanica*, *Vipera lebetina mauritanica*
Daboia palaestinae	*Vipera palaestinae*
Daboia russelii	*Vipera russelii*
Himalayophis tibetanus	*Trimeresurus tibetanus*
Montivipera raddei	*Vipera raddei*
Montivipera xanthina	*Vipera xanthina*
Naja annulata	*Boulengerina annulata*
Naja christyi	*Boulengerina christyi*
Trimeresurus flavomaculatus	*Parias flavomaculatus*
Trimeresurus sumatranus	*Parias sumatranus*
Protobothrops mangshanensis	*Zhaoermia mangshanensis*, *Ermia mangshanensis*, *Trimeresurus mangshanensis*
Trimeresurus stejnegeri	*Viridovipera stejnegeri*

Table A5.2
Changes resulting from new species descriptions or redefinitions (1999–2016)

Currently accepted name	Previous name(s)
Acanthophis crytamydros	Previously part of *Acanthophis rugosus*
Acanthophis laevis	*Acanthophis antarcticus laevis*, confused with *A. antarcticus* or *A. praelongus*

Table A5.2 *continued*

Currently accepted name	Previous name(s)
Acanthophis rugosus (New Guinea)	*Acanthophis antarcticus rugosus*, confused with *A. antarcticus* or *A. praelongus*
Agkistrodon howardgloydi	*Agkistrodon bilineatus howardgloydi*
Agkistrodon russeolus	*Agkistrodon bilineatus russeolus*
Agkistrodon taylori	*Agkistrodon bilineatus taylori*
Bitis gabonica	*Bitis gabonica gabonica*
Bitis harenna	New species
Bitis rhinoceros	*Bitis gabonica rhinoceros*
Bothrops diporus	*Bothrops neuwiedi diporus*
Bothrops mattogrossensis	*Bothrops neuwiedi mattogrossensis*, *B.n. bolivianus*
Bothrops pubescens	*Bothrops neuwiedi pubescens*
Bungarus persicus	New species
Cerrophidion sasai	Previously part of *Cerrophdion godmani*
Cerrophidion wilsoni	Previously part of *Cerrophidion godmani*
Crotalus oreganus	Previously considered part of *Crotalus viridis*
Crotalus ornatus	Previously considered part of *Crotalus molossus*
Crotalus simus	*Crotalus durissus durissus* (Central American populations of *C. durissus* complex)
Crotalus totonacus	*Crotalus durissus totonacus*
Crotalus tzabcan	*Crotalus simus tzabcan*, *Crotalus durissus tzabcan*
Daboia russelii	*Daboia russelii russelii*, *Daboia r. pulchella*
Daboia siamensis	*Daboia russelii siamensis*, *D.r. limitis*, *D.r. sublimitis*, *D.r. formosensis*
Echis borkini	Previously part of *Echis pyramidum*
Echis omanensis	Previously known as NE population of *Echis coloratus*
Gloydius intermedius	Previously named *Gloydius saxatilis*
Hypnale zara	New species

Table A5.2 *continued*

Currently accepted name	Previous name(s)
Lachesis acrochorda	Previously part of *Lachesis stenophrys*
Naja arabica	Previously part of *Naja haje*
Naja anchietae	*Naja annulifera anchietae*, *Naja haje anchietae*
Naja ashei	Previously part of *Naja nigricollis*
Naja nigricincta	*Naja nigricollis nigricincta*, *Naja nigricollis woodi*
Naja nubiae	Previously part of *Naja pallida*
Naja senegalensis	Previously part of *Naja haje*
Pseudechis rossignolii	*Pailsus rossignolii*, previously part of *Pseudechis australis*
Pseudonaja aspidorhyncha	Previously part of *Pseudonaja nuchalis*
Pseudonaja mengdeni	Previously part of *Pseudonaja nuchalis*
Thelotornis mossambicanus	*Thelotornis capensis mossambicanus*
Thelotornis usambaricus	*Thelotornis capensis mossambicanus*
Trimeresurus cardamomensis	Previously part of *Trimeresurus macrops*
Trimeresurus rubeus	Previously part of *Trimeresurus macrops*
Tropidolaemus philippensis	Previously part of *Tropidolaemus wagleri*
Tropidolaemus subannulatus	Previously part of *Tropidolaemus wagleri*
Vipera renardi	Previously part of *V. ursinii*
Walterinnesia morgani	Previously part of *Walterinnesia aegyptia*

7.2 Medically important venomous snakes

Based on current herpetological and medical literature, it is possible to partially prioritize the species of snakes that are of greatest medical importance in different regions. Detailed statistics on the species of snakes responsible for morbidity and mortality throughout the world are lacking, except for a few epidemiological studies which include rigorous identification of the biting snake in a few scattered localities. Thus, establishing a list of medically important species for different countries, territories and other areas relies, at least in part, on extrapolation from the few known studies, as well as on the biology of the snake species concerned: for example, where species of a group of snakes are known to be of public health

importance, based on epidemiological studies, it seems reasonable to deduce that closely related species with similar natural history occurring in hitherto unstudied regions are also likely to be medically important. Examples include Asian cobras in several under-studied regions of Asia, lowland *Bungarus* species in Asia, and spitting cobras in Africa.

Tables A5.3–A5.6 list the species of venomous snakes of greatest medical importance in each of four broad geographical regions. Species listed in these tables are either:

- those that are common or widespread in areas with large human populations and which cause numerous snake-bites, resulting in high levels of morbidity, disability or mortality among victims; or
- poorly known species that are strongly suspected of falling into this category; or
- species that cause major and life-threatening envenoming responsive to antivenom, but are not common causes of bites.

The venoms of these species should be considered a starting point for establishing the most important targets for antivenom production. The need for additional epidemiological and toxinological research to better define which venoms to include and exclude for antivenom production in various regions, territories and countries around the world is emphasized. Detailed data from countries, territories and other areas on species believed to contribute most to the global burden of injury, and/or which pose the most significant risk of morbidity or mortality are provided in Appendix 1 of these Guidelines. Illustrations of some important venomous snakes of Africa and the Middle East are shown in Figs A5.1 and A5.2.

Table A5.3
Medically important venomous snakes: Africa and the Middle East

North Africa/Middle East
Atractaspididae: *Atractaspis andersonii*; Elapidae: *Naja arabica*, *Naja haje*, *Naja oxiana*; Viperidae: *Bitis arietans*; *Cerastes cerastes*, *Cerastes gasperettii*; *Daboia mauritanica*, *Daboia palaestinae*; *Echis borkini*, *Echis carinatus*, *Echis coloratus*, *Echis omanensis*, *Echis pyramidum*; *Macrovipera lebetina*; *Montivipera xanthina*; *Pseudocerastes persicus*
Central sub-Saharan Africa
Elapidae: *Dendroaspis jamesoni*, *Dendroaspis polylepis*; *Naja anchietae*, *Naja haje*, *Naja melanoleuca*, *Naja nigricollis*; Viperidae: *Bitis arietans*, *Bitis gabonica*, *Bitis nasicornis*; *Echis leucogaster*, *Echis ocellatus*, *Echis pyramidum*

Table A5.3 *continued*

Eastern sub-Saharan Africa
Elapidae: *Dendroaspis angusticeps*, *Dendroaspis jamesoni*, *Dendroaspis polylepis*; *Naja anchietae*, *Naja annulifera*, *Naja ashei*, *Naja haje*, *Naja melanoleuca*, *Naja mossambica*, *Naja nigricollis*; Viperidae: *Bitis arietans*, *Bitis gabonica*, *Bitis nasicornis*; *Echis pyramidum*
Southern sub-Saharan Africa
Elapidae: *Dendroaspis angusticeps*, *Dendroaspis polylepis*; *Naja anchietae*, *Naja annulifera*, *Naja mossambica*, *Naja nigricincta*, *Naja nivea*; Viperidae: *Bitis arietans*
Western sub-Saharan Africa
Elapidae: *Dendroaspis jamesoni*, *Dendroaspis polylepis*, *Dendroaspis viridis*; *Naja haje*, *Naja katiensis*, *Naja melanoleuca*, *Naja nigricollis*, *Naja senegalensis*; Viperidae: *Bitis arietans*, *Bitis gabonica*, *Bitis nasicornis*, *Bitis rhinoceros*; *Cerastes cerastes*; *Echis jogeri*, *Echis leucogaster*, *Echis ocellatus*

Table A5.4
Medically important venomous snakes: Asia and Australasia

Central Asia
Elapidae: *Naja oxiana*; Viperidae: *Echis carinatus*; *Gloydius halys*; *Macrovipera lebetina*
East Asia
Elapidae: *Bungarus multicinctus*; *Naja atra*; Viperidae: *Trimeresurus albolabris*; *Daboia russelii*; *Deinagkistrodon acutus*; *Gloydius blomhoffii*, *Gloydius brevicaudus*; *Protobothrops flavoviridis*, *Protobothrops mucrosquamatus*; *Trimeresurus stejnegeri*
South Asia
Elapidae: *Bungarus caeruleus*, *Bungarus ceylonicus*, *Bungarus niger*, *Bungarus sindanus*, *Bungarus walli*; *Naja kaouthia*, *Naja naja*, *Naja oxiana*; Viperidae: *Trimeresurus erythrurus*; *Daboia russelii*; *Echis carinatus*; *Hypnale hypnale*; *Macrovipera lebetina*
South-East Asia (excluding Indonesian West Papua)
Elapidae: *Bungarus candidus*, *Bungarus magnimaculatus*, *Bungarus multicinctus*, *Bungarus slowinskii*; *Naja atra*, *Naja kaouthia*, *Naja mandalayensis*, *Naja philippinensis*, *Naja samarensis*, *Naja siamensis*, *Naja sputatrix*, *Naja sumatrana*; Viperidae: *Calloselasma rhodostoma*; *Trimeresurus albolabris*, *Trimeresurus erythrurus*, *Trimeresurus insularis*; *Daboia siamensis*; *Deinagkistrodon acutus*

Table A5.4 *continued*

Australo-Papua (includes Indonesian West Papua)
Elapidae: *Acanthophis laevis*; *Notechis scutatus*; *Oxyuranus scutellatus*; *Pseudechis australis*;[3] *Pseudonaja affinis*, *Pseudonaja mengdeni*, *Pseudonaja nuchalis*, *Pseudonaja textilis*

Table A5.5
Medically important venomous snakes: Europe

Central Europe
Viperidae: *Vipera ammodytes*
Eastern Europe
Viperidae: *Vipera berus*
Western Europe
Viperidae: *Vipera aspis*, *Vipera berus*

Table A5.6
Medically important venomous snakes: the Americas

North America
Viperidae: *Agkistrodon bilineatus*, *Agkistrodon contortrix*, *Agkistrodon piscivorus*, *Agkistrodon taylori*; *Bothrops asper*; *Crotalus adamanteus*, *Crotalus atrox*, *Crotalus horridus*, *Crotalus oreganus*, *Crotalus simus*, *Crotalus scutulatus*, *Crotalus molossus*, *Crotalus viridis*
Caribbean
Viperidae: *Bothrops* cf. *atrox* (Trinidad), *Bothrops caribbaeus* (St Lucia), *Bothrops lanceolatus* (Martinique); *Crotalus durissus* (Aruba)
Central America
Viperidae: *Bothrops asper*; *Crotalus simus*

[3] *Pseudechis australis* is common and widespread and causes numerous snake-bites; bites may be severe, although this species has not caused a death in Australia since 1968.

Table A5.6 *continued*

South America
Viperidae: *Bothrops alternatus*, *Bothrops asper*, *Bothrops atrox*, *Bothrops brazili*, *Bothrops bilineatus*, *Bothrops diporus*, *Bothrops jararaca*, *Bothrops jararacussu*, *Bothrops leucurus*, *Bothrops matogrossensis*, *Bothrops moojeni*, *Bothrops pictus*, *Bothrops venezuelensis*; *Crotalus durissus*; *Lachesis muta*

7.3 Minor venomous snake species

In many countries, territories and other areas there are species of snakes that rarely bite humans but are capable of causing severe or fatal envenoming. Their medical importance may not justify inclusion of their venoms in the immunizing mixture for production of polyspecific antivenoms but the need to make antivenoms against these species needs to be carefully analysed.

In some cases, such as with some Central American pit vipers (genera *Agkistrodon*, *Porthidium*, *Bothriechis*, *Atropoides* among others), there is clinically effective cross-neutralization of venoms by standard national polyspecific antivenoms (*35*).

In other cases, there is no effective cross-neutralization and manufacturers may therefore consider that the production of a monospecific antivenom is justified for use in potentially fatal cases of envenoming, provided that such cases can be identified. Such antivenoms are currently available for envenoming by the boomslang (*Dispholidus typus*), desert black snake (*Walterinnesia aegyptia*), Arabian burrowing asp (*Atractaspis andersonii*) (*36*), king cobra (*Ophiophagus hannah*), Malayan krait (*Bungarus candidus*) (*36*) "yamakagashi" (*Rhabdophis tigrinus*) and red-necked keelback (*R. subminiatus*), Martinique's "Fer-de-lance" (*Bothrops lanceolatus*), St Lucia's *B. caribbaeus*, and some species of American coral snake (*Micrurus*).

No antivenoms are currently available for envenoming by species such as African bush vipers (for example, *Atheris*, *Proatheris*), berg adder (*Bitis atropos*) and several other small southern African *Bitis* spp. (for example, *B. peringueyi*), Sri Lankan and south-west Indian hump-nosed vipers (*Hypnale* spp.) (*37*, *38*), many Asian pit vipers ("*Trimeresurus*" *sensu lato*), some species of kraits (for example, *B. niger*) and all but one species of burrowing asp (genus *Atractaspis*).

An alternative to antivenom production against species that cause few, but potentially severe envenomings, is to manufacture polyspecific antivenoms for broadly distributed groups that have similar venom compositions (for example, African *Dendroaspis* and *Atractaspis*; Asian "green pit vipers"; American *Micrurus*). This may result in antivenoms that offer broad protection against

venoms from minor species within genera, or species whose bites are less frequent than those of others in the same taxonomic groups (that is, genus, subfamily or family).

7.4 Sea snake venoms

Although venomous marine sea snakes have not been included in the tables of medically important venomous snakes, it should be recognized that there are a number of species of marine snakes with potent venoms that can cause illness or death. Available evidence, particularly clinical experience, indicates that the major sea snake antivenom that is currently commercially available, which uses venom of a single sea snake, *Hydrophis schistosus* (previously known as *Enhydrina schistosa*), in the immunizing venoms mixture, is effective against envenoming by other sea snake species for which there are clinical data. Further research would be needed to better define the full extent of cross-neutralization offered by this antivenom against other sea snake species.

7.5 Main recommendations

- **Clinicians, toxinologists, poison centres, regulators, venom producers and antivenom manufacturers should be well informed about current nomenclature and new changes to taxonomy, so as to ensure the currency of information, correct identification of species in their countries, and correct selection and sourcing of venoms used in the manufacture of antivenoms.**
- **Identification of the medically important venomous snakes (those that cause the greatest burden of injury, disability and/or mortality) is a critical prerequisite to meeting the need for efficacious antivenom. Improving the quality of the available data and correcting and amplifying the level of geographical detail and precision of attribution should be important priorities.**
- **Support for establishment of local capacity for venom production as a means of ensuring that venom immunogens from geographically representative populations of medically important snake species are used in antivenom production would improve antivenom specificity.**

Fig. A5.1

Medically important North African and Middle Eastern venomous snakes: (A) Egyptian cobra (*Naja haje*), (B) East Africa carpet viper (*Echis pyramidum*), (C) puff adder (*Bitis arietans*), (D) Saharan horned viper (*Cerastes cerastes*) and (E) Levant viper (*Macrovipera lebetina*)

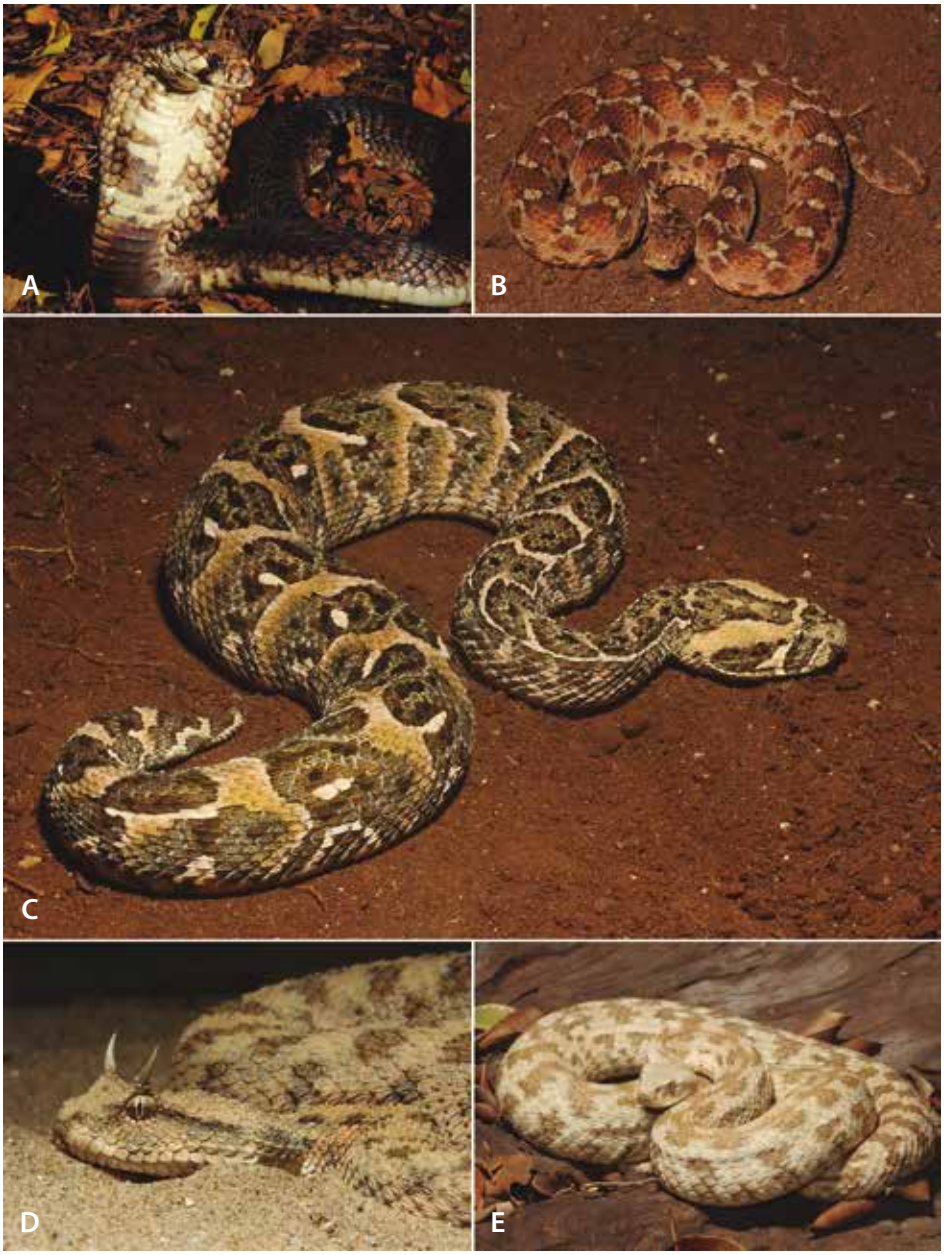
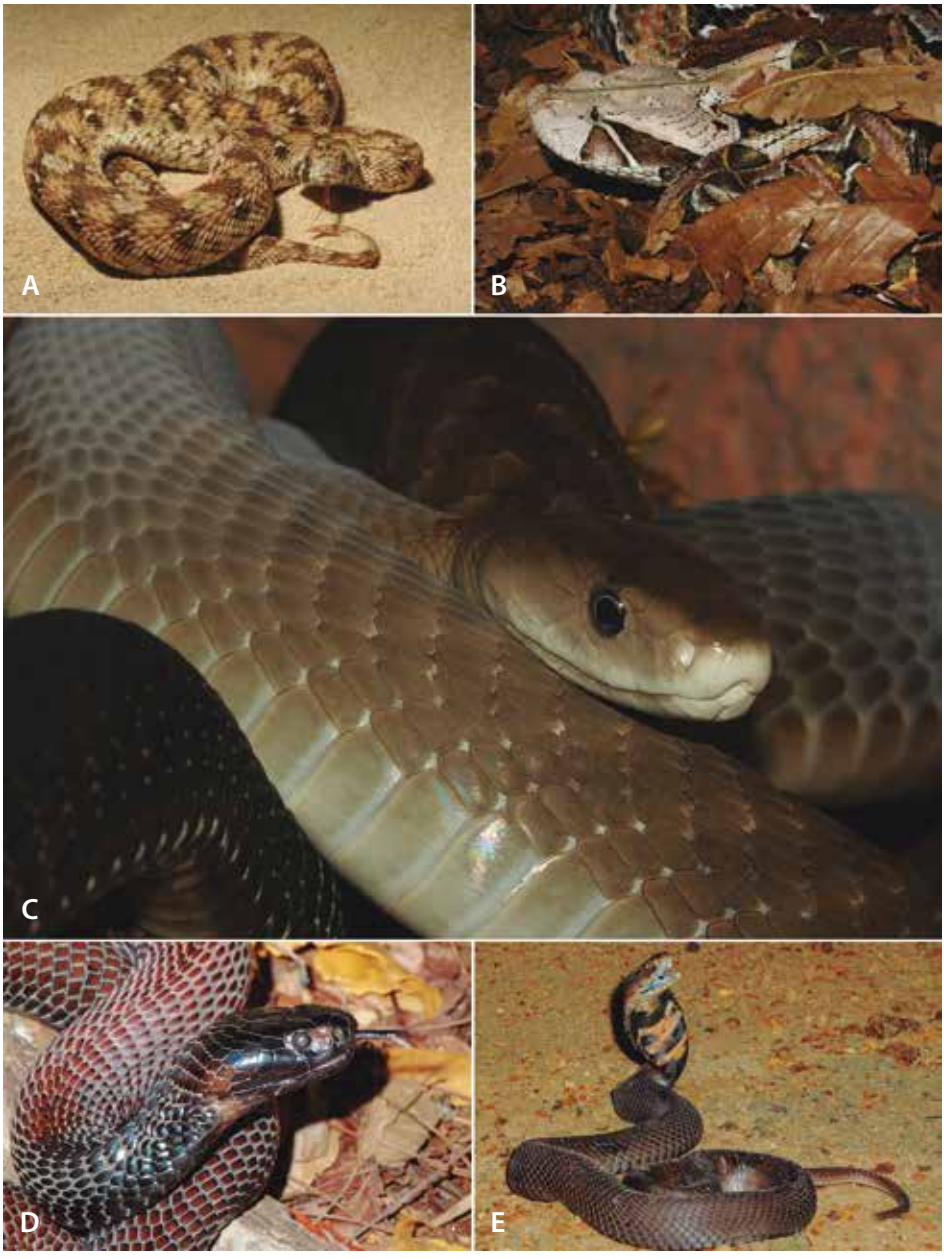


Fig. A5.2
Medically important sub-Saharan African venomous snakes: (A) West African carpet viper (*Echis ocellatus*), (B) Gaboon viper (*Bitis gabonica*), (C) Black mamba (*Dendroaspis polylepis*), (D) Black-necked spitting cobra (*Naja nigricollis*), (E) Mozambique spitting cobra (*Naja mossambica*)



8. Antivenoms design: selection of snake venoms

Venomous snakes exhibit significant species- and genus-specific variation in venom protein composition (*39*). The clinical effectiveness of antivenom is therefore largely restricted to the venom(s) used in its manufacture. It is therefore imperative that antivenom manufacturers carefully consider the venoms used in antivenom manufacture by first defining the geographical area where the antivenom will be deployed, and sequentially:

- identifying the most medically important snakes in that region;
- examining the venom protein composition of the snakes, including information from relevant literature;
- conducting antivenom preclinical efficacy tests on venoms of all the most medically important snakes in that region.

8.1 Selection and preparation of representative venom mixtures

Appendix 1 presents an up-to-date list of the most medically important venomous snake species by country, region and continent. The venoms from Category 1 snakes must be included for antivenom production and venoms from Category 2 snakes only excluded after careful risk–benefit assessment.

It is important to appreciate that there are variations in venom composition and antigenicity: (a) within the geographical range of a single species; and (b) between snakes of different ages (*40*, *41*). Therefore, venom should be collected from specimens of different geographical origins and ages, and mixed before being used for immunization (see section 9 on venom preparation). The greater the intra-specific variation, the more snake specimens of distinct origin and age are required to create an adequate venom immunization mixture.

Cross-neutralization of venoms with similar protein composition profiles to the venoms used for immunization may extend the effectiveness of some antivenoms, but requires, minimally, preclinical efficacy testing to identify the potential cross-neutralization capacity of an antivenom. In vitro preclinical immunological cross-reactivity testing alone is NOT an adequate measure of antivenom efficacy.

8.2 Manufacture of monospecific or polyspecific antivenoms

Antivenom manufacturers face an early, critical decision as to whether the antivenom should possess monospecific or polyspecific effectiveness.

8.2.1 Monospecific antivenoms

Monospecific antivenoms are manufactured with venoms from a single venomous snake species, and their effectiveness is largely restricted to that snake species. These conditions apply in areas where:

- there is only one medically important species (for example, *Vipera berus* in Scandinavia and the United Kingdom) or where one species is responsible for the majority of cases (for example, *Oxyuranus scutellatus* in southern Papua New Guinea);
- a simple blood test, suitable for use even in under-resourced health-care centres, can define the biting species (for example, detection of incoagulable blood by the 20-minute whole blood clotting test in the northern third of Africa, where only *Echis* spp. cause coagulopathy);
- a simple algorithmic approach allows the species to be inferred from the pattern of clinical and biological features;
- there is a reliable and affordable rapid immunodiagnostic test readily available allowing the toxins to be identified unambiguously (currently only available in Australia).

Monospecific antivenoms can be effective in treating envenoming by a few closely related species whose venoms show clinically effective cross-neutralization – but this requires preclinical and clinical confirmation.

8.2.2 Polyspecific antivenoms

Most tropical countries are inhabited by several medically important snake species, and it is commercially unrealistic to develop multiple monospecific antivenoms. In these cases, the manufacture of polyspecific antivenoms is highly recommended. Polyspecific antivenoms are designed to contain IgG effective against venoms from multiple species or genera of venomous snakes in a defined region. Manufacturing protocols of polyspecific antivenom include:

1. Mixing venoms from multiple snake species or genera (sometimes in amounts quantitatively associated with medical importance, immunogenicity etc.) and immunizing donor animals with this mixture. Immunizing an animal with venoms from several taxonomically related snakes (for example, different vipers) can have the advantage over monospecific antivenom of increasing the titre of neutralizing IgG to any one snake venom (*42*).

2. Immunizing groups of donor animals with distinct venom mixtures and then mixing the hyperimmune plasma from each group of animals.
3. Immunizing groups of donor animals with distinct venom mixtures and then mixing the monospecific antivenom IgGs to formulate the final polyspecific antivenom.

When using options 2. and 3. it is important to monitor the efficacy for each monospecific antivenom to ensure that the efficacy of the mixed final product is consistent, reproducible and in line with the product specification for each individual venom. This "combined monospecific antivenoms" approach anticipates that the amount of neutralizing IgG targeting each individual venom will be proportionally diluted – necessitating administration of more vials to reverse venom pathology, which in turn increases the risks of adverse reactions.

In some regions, it is possible to differentiate envenoming by detecting distinct clinical syndromes: neurotoxicity, haematological disturbances (haemorrhage or coagulopathy) and/or local tissue damage. Such situations justify the preparation of syndrome-specific polyspecific antivenoms by immunizing donor animals with mixtures of either neurotoxic venoms or venoms causing haemorrhage and/or coagulopathy and local tissue damage.

In most tropical regions where snake-bite is a significant medical burden, polyspecific antivenoms offer significant clinical advantages and their production should be encouraged. They can also offer greater commercial manufacturing incentives (economies of scale) than monospecific antivenoms because of their significantly greater geographical and snake-species cover – increasing the likelihood of their delivery to victims residing in regions where antivenom manufacture is not government subsidized.

8.3 Main recommendations

- **Prior to importing antivenoms, national health authorities should carefully consider their regional threat from venomous snakes to guide their antivenom requirements.**
- **The design of the venom mixture used in immunization, and the decision to prepare monospecific or polyspecific antivenoms, must be guided by the epidemiological and clinical information on snake-bites in the defined country, region or continent.**
- **In most tropical countries polyspecific antivenoms are likely to have significant clinical and logistical advantages over monospecific antivenoms, particularly in the absence of rapid, affordable snake venom diagnosis.**

- **Polyspecific antivenom may be prepared from IgG of donor animals immunized with a mixture of venoms, or by mixing monospecific antivenoms.**
- **Manufacturers seeking marketing authorization for antivenoms in a given country should provide experimental evidence from preclinical testing that the product exhibits a neutralization capacity against different local venoms (see section 19).**
- **National health authorities should organize independent preclinical efficacy testing prior to importation of any antivenom to avoid national distribution of dangerously ineffective products.**

9. Preparation and storage of snake venom

Venom preparations are used both to hyperimmunize animals as part of antivenom production, and to provide reference venom samples for routine and/or preclinical potency assessment of antivenoms. According to GMP for pharmaceutical products, snake venoms are starting materials, and therefore ensuring their quality is critical, and their preparation should follow the principles and recommendations stated below. The essential principles of quality systems should be applied to venom production including traceability, reproducibility, taxonomic accuracy and hygiene control. Manufacturers of snake venoms used in antivenom production should strive to comply with WHO's Guidelines on GMP for biological products and Guidelines for good manufacturing practices for pharmaceutical products.[4]

Venoms used for antivenom manufacture should be representative of the snake population living in the area where the antivenom is to be used. To take account of the variability in venom composition within a species (*43–47*), it is imperative that the venom of an adequate number of individual snakes (generally no fewer than 20 specimens, including males and females) collected from various regions covering the entire geographical distribution of the particular venomous snake species should be collected together. Consideration should also be given to including venom from juvenile or sub-adult snakes in these venom pools as there is strong evidence of age-related venom variation within individual specimens and populations (*48*). A similar approach should be used in the preparation of Standard Reference Venoms (national or regional) for use in the validation of antivenom products by reference laboratories and

[4] WHO Good Manufacturing Practices for Biological Products. WHO Technical Report Series, No. 996, 2016, Annex 3. (Replacement of Annex 1 of WHO Technical Report Series, No. 822).

regulatory agencies (see section 10) or in preclinical testing of antivenoms by manufacturers (see section 19).

Venom producers should ensure that they fully document, and can provide evidence of:

- geographical origin and the length or age (juvenile or adult) of each individual snake used for venom production;
- taxonomic details of each snake species used;
- correct implementation of compliance with local wildlife legislation, and the Convention on International Trade in Endangered Species (CITES) documents in the case of endangered species;
- application of appropriate withholding rules (for example, not collecting venom from animals under quarantine, or which are gravid, injured, sick or in poor condition);
- individual identification of snake specimens contributing to each venom batch;
- traceability of each venom batch;
- appropriate handling and stabilization of venoms (for example, rapid freezing of the venom after collection and lyophilization for long-term stable storage);[5]
- quality control confirmation of batch-to-batch consistency of venoms of each species and country of origin (for example, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or high-performance liquid chromatography (HPLC) profiling of venoms, measurement of residual moisture in lyophilized venom);
- confirmation of batch-to-batch similarity of venom of the same origin.

9.1 Production of snake venoms for immunization

The maintenance of a serpentarium and the handling of snakes used for antivenom production should comply with quality systems principles.

9.1.1 Quarantine of snakes

All new accessions should be quarantined for at least 2 months in a special "quarantine room" which should be located as far as possible from the "production rooms" where snakes qualified for venom production are kept.

[5] Desiccation or vacuum-drying may be acceptable if proven to ensure stability of the preparation.

On arrival, snakes should be examined by a specialized veterinary surgeon (or experienced person) for ectoparasites, wounds and fractures. Endoparasites (nematodes, cestodes, trematodes and pentastomids) should be eliminated using broad-spectrum antiparasitic drugs and any injury must be adequately treated by a veterinarian (*49–51*). Some viruses can be transmitted between different species, and between different families of snakes. Therefore, different families should be kept in different rooms.

Sick snakes should be treated and their quarantine extended for 1–2 months after complete clinical recovery. Sick animals found in "production rooms" may be treated in situ (although quarantine is preferable) but they cannot be used for venom production. If an antibiotic treatment is given, the snake should not be used to obtain venom for 4 weeks following the end of the treatment. When housed in good conditions, adult snakes collected from the wild can live in captivity for 10 years or more. When handling snakes, the risk of infection with human mosquito-borne viruses such as Japanese encephalitis should be prevented, since arbovirus infections have been reported in some snakes (*52*).

9.1.2 Maintenance of captive snakes for venom production

Individual snakes should preferably be housed in separate cages large enough to allow them to move about, according to local and international standards. There are several acceptable options for the design of the cages. Transparent or black (for burrowing snakes) plastic boxes are recommended. Cage materials should be impermeable, free from fissures, and inert to disinfectants, cleaning chemicals and common solvents. Cleaning and disinfecting agents should be carefully selected to ensure they do not have adverse effects on the snakes. Cages should be adequately ventilated but perforations or mesh must be small enough to prevent escape. Ventilation holes should be clearly marked as hazard areas since there is a risk of accidental envenoming (for example, spitting cobras have been known to spray venom through such openings, and large vipers have fangs which can extend through a small hole if the snake strikes). In the case of gravid viviparous snakes, the ventilation holes or mesh should be sufficiently fine to prevent escape of their tiny, liveborn offspring. The cage interior should be visible from the outside to allow safe maintenance and handling. Access to cages through doors, lids or sliding panels should facilitate management without compromising safety or allowing snakes to escape. Be wary of cages with internal ledges or lips above doors, as some snakes can conceal themselves above them out of sight of the keepers. A disposable floor covering (for example, newspaper) is recommended. Cryptic and nocturnal species should be provided with a small shelter where they can hide.

The use of "hide boxes" is increasingly common as these provide both a more reassuring environment for the snake, and increased safety for keepers.

Hide boxes should be designed to be slightly larger than the curled snake, with an entrance/exit hole large enough to allow a recently fed snake easy access, plus some simple closure device to lock the snake in the hide box. This will allow removal of the snake from the cage without hazard to the keeper, making routine cage maintenance simpler and safer. Hide boxes can be made from plastic, wood or even cardboard (which is inexpensive and can be discarded and replaced regularly). Permanent hide boxes should be readily cleanable or autoclavable. The roof or side of the hide box should be removable, to allow easy and safe extraction of the snake when required.

Cages should be thoroughly cleaned and disinfected when soiled (daily if necessary). Faeces and uneaten or regurgitated food items should be removed as soon as possible. To avoid misidentification of the snake, a microchip should be implanted in the hypodermal layer of the snake's posterior region and a label bearing its individual data should be attached to the cage and transferred with the snake when it is moved to another cage. Water should be freely available, and for species from humid climates more frequent watering or misting may be required, particularly when sloughing. Water should be changed regularly and as soon as it becomes contaminated. Water treatment by ultraviolet (UV) sterilization or acidification may be considered.

Tens of cages may be accommodated in the same "production room", provided that there is enough space for maintenance and venom extraction. This room should be kept as clean as possible at all times, and thoroughly cleaned at least weekly. Measures should be taken to minimize or eliminate contamination or spread of diseases. The use of antiseptic hand washes, disposable over-clothing, antiseptic foot wash trays at entry and exit points, and other measures should be routine. The temperature and humidity of the snake room should be controlled according to the climatic requirements of the particular snake species. Ventilation should be ensured using fans, air-conditioning, or air renewing systems.

Access to snake rooms should be restricted to personnel responsible for their maintenance. The rooms should be kept locked, with any windows permanently closed or protected by bars and mosquito proofing. Access should be via a safety porch not allowing simultaneous door opening and with a transparent panel allowing a view of the entire snake room for pre-entry safety inspections. The spaces below the doors should be less than 3 mm and all openings to the exterior (for example, water pipes, drainage conduits, ventilation entrances and exits) should be protected by grilles having holes smaller than 3 mm. Natural light is often used; however, when not available, artificial light should be turned on for 12 hours during the day and turned off during the night for tropical species, but species from temperate zones may have different requirements. Snakes of the same species, collected at the same time in the same area should be placed in the same racks. The same "production room"

can contain snakes of different species, provided that they require similar living conditions (that is, temperature and humidity).

When kept under favourable housing and climatic conditions, and if left undisturbed, snakes will reproduce in captivity (*53*). Animals should be mated only with specimens from the same species, subspecies and local origin (*54*, *55*). Sexing can be difficult, but is helped by the use of intra-cloacal probes. The male and the female should be individually identified and separated soon after copulation. The female should be kept under careful surveillance. Eggs from oviparous snakes and neonates from viviparous snakes should be removed from the female's cage as soon as possible. Differences in the venom composition of adult and juvenile snakes have been reported in some species (*43*, *48*, *56–58*), and where this is known to occur or is suspected, the venom of a certain proportion of juvenile snakes might be mixed with that of adults during the production of venom batches.

The ideal frequency of feeding captive snakes depends on the species and age of the snake, varying from twice per week to once per month. Snakes are usually fed after venom extraction, ideally with dead mice or other appropriate prey according to the snake species. Animals such as rats and mice that are raised to feed snakes should be produced under appropriate quarantine standards in facilities designed for this task. Humane euthanasia should be employed in the killing of food animals, and ideally these food animals should be frozen for at least 7 days before being thawed for use. Some snakes will only accept living prey, but attempts should be made to wean them onto dead prey, and all local ethical standards should be followed in the production and use of food animals. Snake-eating species, such as kraits, coral snakes and king cobras, can be enticed to take dead mice if the prey is first flavoured with snake tissue fluids, although any such material should be frozen first for at least 7 days to kill parasites, before it is thawed for use. Some coral species can be fed with fish strips (*59*). Living, dead or regurgitated prey should not be left in the cage for more than a few hours. Force feeding may be necessary for neonates and snakes that persistently refuse to feed. Feeding time affords an opportunity to carefully check the snake for abnormal behaviour, wounds, and possible infections and to give dietary supplements when necessary. Individual feeding records are crucial. They should include details of what, when and how prey was offered, when it was consumed and whether it was regurgitated. The health of captive snakes can be estimated and recorded by observing regular feeding and by measuring their weight and length. These data are best stored on a computer system, using a "barcode" for each snake, or, alternatively, using a reliable manual recording system, and constitute useful records related to the venom batches produced. Venom extraction rooms should be equipped with emergency eyewash stations and safety showers as is the case in laboratories where there is a risk of chemical contact hazards.

9.1.3 General maintenance of a serpentarium

Serpentariums should be designed to comply with appropriate GMP principles. Quarantine facilities should be isolated in all respects from the main animal housing area, and should have separate air-handling systems, or be in a separate building. Maintenance areas such as storerooms, rooms for cleaning and sanitizing cages and racks, animal houses for production of food animals (for example, rodents or invertebrates), and rooms used for administration or for venom processing, venom quality control and secure storage of venoms, should also be separated by appropriate barrier systems from the main snake housing and venom extraction rooms. The main housing rooms for snakes used in venom production should be designed with security, hygiene and disease control needs in mind. Separate rooms for accommodation of snake egg incubators and both neonates and juvenile snakes should be included in the design of the serpentarium.

The cage cleaning rooms should be large enough to hold all the cages that are being cleaned and sanitized. Dirty cages and other items should be kept separate from clean cages and equipment being stored ready for use. Furthermore it is desirable to have two sets of washing and sanitizing rooms, a larger one for equipment from the venom production room and a smaller one for equipment from the quarantine area. These rooms should be secure in case a snake is inadvertently left in its cage when the container is placed in the cleaning room. The cleaning procedures for production rooms and for cages in which snakes are kept, and the cleaning schedule, should be established and documented.

Food animals, usually rodents, should be purpose bred in clean conventional animal houses, and kept, handled and killed in accordance with ethical principles. The rooms, exclusively used for rodent production, should be large enough to provide sufficient numbers of rats or mice to feed the snakes. Ideally, rodent production should be performed in facilities meeting the corresponding international guidelines. Alternatively, rodents can be purchased from qualified commercial sources. Breeding of rats and mice cannot take place in the same room, because of the stress induced by the rats in the mice. The diets required by young snakes may differ from those of adults (for instance, frogs and tadpoles are preferred to rodents by some species), and facilities for producing these food animals may also be required.

When possible, it is useful to have a small laboratory for performing quality control on the venoms. All serpentariums need to be designed with separate laboratories where venom can be processed after extraction and quality control performed (see section 10). An area for repairing broken equipment and for other miscellaneous purposes is also required. The administrative area should be sufficiently large and adequately equipped with computer facilities so that the traceability requirements needed for venom production can be met. The whole venom production facility should be made secure against unauthorized intrusion.

9.1.4 Snake venom production

The collection of venom is an inherently dangerous task; therefore specific safety protocols for operators must be applied and rigidly enforced (see section 9.2). All operations should be fully described in written procedures and SOPs, which should be checked and revised periodically according to a written master document. Pools of venom require unique batch numbers, and should be traceable to the individual specimens from which venom was collected for that batch.

9.1.4.1 Venom collection in serpentariums

Venom can be extracted from snakes according to a regular schedule, depending on the species. The interval between extractions varies among producers and ranges from every 2 or 3 weeks to every 3 months. Specimens that are quarantined, are gravid, are undergoing treatment for sickness or injury, or in the process of sloughing their skins should not be used for venom production.

Handling equipment must be appropriate for the particular species of snake to minimize risk of stress, discomfort and injury to both the snake and the operator. Staff must be familiar with the equipment and properly trained in its use. Common methods of restraint include gently removing the snake from its cage with a hook and either placing it on a foam rubber pad before being pinned behind the head, or encouraging the snake to crawl into a transparent plastic tube in which it can be restrained. Developing innovative methods that enable safe restraint of venomous snakes and minimize the risk of injury both to operators and snakes is strongly recommended. For very dangerous species, the use of short-acting general anaesthesia or moderate cooling (15 °C) during venom extraction can be considered (for example, inhaled isoflurane or sevoflurane or even carbon dioxide) as it reduces the risk of accidents both to the snake and to the snake handler. Excessive cooling of the snake in a refrigerator is potentially harmful and is not recommended. For the collection of venom, the snake's head is grasped in one hand just behind the angle of the jaw, while the snake's body is held with the other hand, or by an assistant snake handler. Individual techniques for holding the head of the snake vary and each operator should use the method that works best for them. An assistant should gently occlude the snake's cloaca to prevent messy contamination of the locality by spraying of faeces.

Different techniques are used to collect venom. Many rely on encouraging the snake to open its mouth and either bite through a plastic- or parafilm-covered membrane, which provides a barrier to contaminants such as saliva and blood (from minor oral trauma), or to release venom into a container over which the fangs have been hooked by the operator. In the case of large vipers, the dental sheath may be retracted when necessary with sterile forceps. Although it is common practice to squeeze the sides of the snake's head to try to force venom

from the glands, this may cause traumatic bruising to the animal and should be avoided. The use of brief electrical impulses of moderate intensity to stimulate venom secretion is not recommended. Any venom sample contaminated with blood should be centrifuged. After venom extraction, the fangs are carefully withdrawn from the collection vessel, while preventing damage to the mouth and dentition and avoiding the snake impaling itself with its own fangs. Then, the oral cavity should be sprayed with an antiseptic solution to avoid stomatitis. After each venom extraction, all materials used in the process should be sterilized.

Peptides and proteins in venom are amphiphatic and will adhere to most common surfaces including glass and plastic (*60*) resulting in the potential loss of toxins from the venom used to produce hyperimmune plasma. The use of polypropylene vessels and the addition of 1% bovine serum albumin can help reduce such losses, but different peptides may have variable affinity for being retained on vessel surfaces regardless of the approach taken to minimize loss.

Special procedures that avoid direct handling should be employed in the case of burrowing asps (genus Atractaspis) because they cannot be held safely in the way described above (*61*). For some species with small fangs and small venom yields, the use of sterile pipette tips or capillary tubes which are slipped over each fang one at a time, and pressure applied to the base of the fang to stimulate venom release into the tube, is recommended. In the case of colubrid snakes, special techniques are required, such as application of foam rubber pads (from which venom is recovered in the laboratory) or pipette tips/capillary tubes to the posteriorly placed fangs and the use of secretagogue drugs. Similarly, some elapid snakes have small fangs and the pipette tip or capillary tube technique is required to collect venom. At the time of venom extraction, there is an opportunity to remove broken or diseased fangs and to examine the snake for ectoparasites (for example, ticks and mites), wounds, dermatitis, areas of adherent dead skin and retained spectacles over the eyes. The snake can be treated with drugs and/or vitamins at the same time and, if necessary, can be force fed. When force fed with rodents, the rodent's incisors must be cut out so as not to cause any injury to the snake's oesophagus. The process of venom extraction is often combined with cage cleaning and disinfection and the feeding of the snake. Avoiding trauma to the snake's mouth and dentition is critical to prevent infection and "mouth rot" and the venom extraction process should be performed in accordance with clean practices.

Several snakes from the same group (same species and subspecies collected at the same time in the same area) can have their venom collected into the same venom collection vessel. The vessel should be kept in an ice bath between individual extractions, and the venom aliquoted into labelled storage tubes or vials and snap-frozen at −20 °C or colder within 1 hour. For venoms with high proteolytic activity, the collected venom pool should be transferred into a vial maintained at ultra-low temperature (−70 to −80 °C) or at least

−20 °C, every 10–30 minutes, before continuing extractions from that group of specimens. Another method is to transfer the collected venom into a vial maintained in an ice bath. Refrigerated centrifugation of freshly collected venom is recommended, for instance at 1000 g for 5 minutes (4 °C), to remove cellular debris.

It is important to identify the vial into which the venom has been collected or transferred for storage, with an appropriate reference number. Primary indelible identification must be on the vial. This allows the identification of all the snakes used during venom extraction, the name of the operator and any other relevant information. To obtain large venom batches for the preparation of antivenom, especially from species with low yields, one approach is to use the same vial over several months for extractions performed with the same specimens, providing the cold chain is never broken. Pools of venom require unique batch numbers, and the individual venom extractions contributing to the pool must be traceable. When a pool is sufficient in volume, the venom should be either freeze- or vacuum-dried and kept in the dark at a low temperature (either −20 °C or 4 °C) in a well-sealed flask, precisely identified with a number, up to the time of delivery. Some producers use an alternative system, keeping dried venom at 20–25 °C in a desiccator. Regardless of the method used, the procedures for drying venom should be well established, documented, validated and incorporate appropriate quality control steps (for example, periodic determination of residual moisture against established standards). The potency of venom stored for considerable periods of time must be tested at least annually to ensure that no degradation or loss of activity has occurred (see section 10), and if a loss of potency is observed the batch must be replaced.

The equipment used for storage of frozen venom (freezers) and for venom drying, should be cleaned using established procedures, and the cleaning documented, in order to minimize cross-contamination. Likewise, equipment requiring calibration, such as freezers, balances and freeze-driers, should be calibrated according to a defined schedule.

9.1.4.2 Venom collection from wild snakes

The practice of collecting venoms from wild-caught snakes that are subsequently released in either the same or a different location should be discontinued, and is not recommended due to the lack of traceability and difficulties in ensuring effective quality control of venoms. There is also evidence of high levels of mortality among relocated snake species particularly if they are released at a distance from the capture site (*62–65*). In jurisdictions where it is current practice for collectors to go to designated localities in the wild, catch snakes and collect venom before releasing them elsewhere, strong efforts must be made to replace this approach with regulated production using captive snakes maintained in well-designed serpentariums.

9.2 Staff responsible for handling snakes

9.2.1 Safety and health considerations

Handling and extracting venom from snakes is a dangerous operation. One envenoming occurred every two years in each of the 15 extraction facilities reviewed by Powell et al. (*66*). At a commercial venom production plant in Uberlândia, Brazil between 1981 and 1999, 25 technicians performed 370 768 venom extractions from *Bothrops moojeni*. Twelve bites were recorded, 10 with envenoming, and one case of venom squirted into the eye of a worker (*67*).

Venom extractions should be performed according to well-designed and documented SOPs by well-trained snake handlers. All personnel involved in snake handling and venom collection should be fully informed about the potential dangers of being bitten and envenomed. They should be thoroughly trained, and the training procedures must be documented and specific protocols practised as a team. A minimum of two people should be present during snake handling for venom collection. For safety reasons, it is recommended that venom extraction sessions should be interrupted at least every 2 hours for a rest period, before restarting the process.

Personnel involved in snake handling and venom extraction should observe established hygiene standards (see below) to minimize the impact on snakes and the potential transfer of pathogens between snakes.

9.2.2 Personal protective equipment (PPE) for snake or venom handling

Protective clothing should include appropriate eye protection (safety glasses or face shields), face masks, nitrile gloves and a laboratory coat or gown. Eye protection is especially important when handling spitting elapids capable of squirting their venom. The wearing of puncture-resistant gloves designed to prevent an effective bite is unpopular among many keepers who fear that it impairs manual dexterity and sense of touch, but the use of nitrile gloves is advisable to prevent cross-contamination. Puncture-resistant gloves should be mandatory as protective equipment for assistants helping to restrain or handle snakes during procedures such as venom extraction.

When lyophilized or desiccated venom is being handled, the safety of operators is paramount, since dried venom can easily be aerosolized and affect people through skin breaks, eyes or mucous membranes, or by sensitizing them to the venom (*68*). Appropriate gowning is necessary when handling dried or liquid venom, to prevent contact of the venom with skin or mucous membranes. It is highly recommended that a biological safety cabinet (for example, Class II, B2), be used while handling lyophilized or desiccated venom.

9.2.3 Procedures to be followed if a bite occurs

There are several important measures to be put in place for dealing with a bite (*69*), as described below.

9.2.3.1 Procedures and alarms

Clearly defined, prominently displayed, well-understood and regularly rehearsed procedures should be in place in case of a bite. An alarm should be sounded to summon help, the snake returned safely to its cage or box and the victim should withdraw to an area designated for first aid.

9.2.3.2 First aid protocols

Clearly understandable first aid protocols should be established for each species. These should be available in printed form adjacent to each cage. Immediate application of pressure-immobilization may be appropriate for treating the bites of rapidly neurotoxic elapids. However, the technique is not easy and, if they are to use the method properly, staff will need extensive training and regular practice, and must be provided with the necessary materials (a number of crepe bandages, 10 cm wide × 4.5 m long, and splints). Analgesia should only be provided for pain during the pre-hospital period upon the advice of an attending physician. Provision of appropriate analgesia for first aid should be considered. If venom enters the eyes, immediate irrigation with generous volumes of clean water is an urgent necessity.

9.2.3.3 Hospital admission

As a precaution, all victims of bites, scratches by snakes' fangs or teeth, and those in whom venom has entered the eye, or anyone else suspected of a snake-bite or venom exposure injury (for example, through aerosolized dried venom), should be transferred as quickly as possible to the designated local hospital, by prearranged transport, for medical assessment. It may be helpful to remove from the cage, and take to the hospital with the victim, the label identifying the snake responsible for the bite, so that accurate identification of the snake species and of the antivenom to be administered is ensured. Staff members should wear, or carry a card detailing their personal medical information (including drug allergies) at all times and the card should be taken with them to hospital in the event of an injury. The contact details of a recognized clinical toxinology expert should be included on this card.

It is highly recommended that all serpentariums stock in-date supplies of antivenom appropriate to the species of snakes being held, so that an adequate

supply of the correct antivenom can accompany the victim to hospital. Hospital staff should be warned in advance by telephone of the arrival of the casualty and informed about the species responsible and any background medical problems and relevant medical history, such as past reactions to antivenom or other equine sera (for example, anti-tetanus serum), and known allergies.

9.2.3.4 Snake venom hypersensitivity

Snake venom hypersensitivity is an occupational hazard of snake handlers that occurs due to sensitization to venom proteins. Two out of 12 snake-bites in a commercial venom production plant in Brazil resulted in venom anaphylaxis (*67*). Hypersensitivity is usually acquired by mucosal contact with aerosolized dried venom. Important early evidence of evolving sensitization is sneezing, coughing, wheezing, itching of the eyes or weeping when working around snakes and snake enclosures, or even upon entering the snake room. No one with established venom allergy should be permitted to continue working with snakes. Venom-induced anaphylaxis should be treated with self-injectable adrenaline (epinephrine) 0.5 ml of 0.1% solution by intramuscular injection (adult dose), which should be stocked in adequate doses in each room holding snakes, or where snakes are used for procedures such as venom extraction.

9.2.3.5 Medico-legal and health insurance aspects

The occupational exposure to venomous snake-bites in commercial venom production units is the responsibility of the employers and requires their formal attention.

9.3 Main recommendations

- **Well-managed serpentariums are a key element in the production of venom preparations meeting the quality requirements for the production of effective antivenoms.**
- **The quality of snake venoms used for animal immunization, as material for both preclinical and batch release assessment of neutralization efficacy, or for the development of national or regional reference preparations is of critical importance.**
- **The procedures used in snake maintenance, handling and venom extraction, as well as in all aspects of venom collection should be properly documented and scheduled.**
- **Venoms used for antivenom preparations should be representative of the entire snake population living in the area for which the polyspecific and/or monospecific antivenoms are intended to be used. Because of regional and individual variations in**

venom composition within snake species, the venoms used for immunization should be collected from a large number of individuals (generally at least 20, including males and females of different ages) collected from various regions covering the entire geographical distribution of the particular venomous snake species.

- **Venom producers should adhere rigorously to the following recommendations and should be able to demonstrate their application:**
  - **Taxonomic identity and geographical origin of each individual animal used for venom production should be known and recorded.**
  - **Housing, feeding and handling of snakes should meet the highest veterinary and ethical standards, and follow documented protocols.**
  - **Adequate training should be provided to personnel involved in venom production in all procedures, and implementation of health and safety measures.**
  - **Formal guidelines and procedures for emergency response in the event of any suspected snake-bite or venom exposure should be established and well documented.**
  - **Venom should not be collected from animals under quarantine, or which are gravid, injured, sick or in poor condition.**
  - **Full traceability of each venom batch should be ensured.**
  - **Venoms should be frozen as soon as possible after collection, and at least within 1 hour.**
  - **Freeze-drying or desiccation of the venoms should be done under conditions that ensure stability for long-term storage.**
  - **Batch-to-batch consistency of venoms of the same origin should be confirmed.**

10. Quality control of venoms

10.1 Records and traceability

It is critical to accurately identify the species (and subspecies, if any) of each individual snake used for venom production and the taxonomic status should be validated by a competent herpetologist. Increasingly, DNA taxonomy is replacing conventional morphological methods, but this technique is impracticable in most venom production units which will continue to rely on well-established physical features such as colour pattern and scale count to distinguish the principal medically important species.

Internationally recognized scientific names should be used and the biogeographical origin of each snake should be specified, since differences in venom composition may occur between different populations of the same species or subspecies (*43–48*, *70*). Venom producers can consult academic zoologists who have appropriate skill and experience.

Data pertaining to each numbered venom batch should include the information considered to be key for traceability, quality and specific characteristics of the venom (for example, identification of all the snakes used, the species, subspecies and biogeographical origin, feeding, health care, date of each extraction, number of specimens used to prepare the batch, and quantity of venom produced). This information should be made available upon request to any auditor or control authority.

For long-term storage, venom should be appropriately aliquoted to minimize wastage and must then be stored in sealed vials until use. Liquid venoms should be stored frozen at −80 °C, while lyophilized or dried venoms may be stored at −20 °C. After opening the vial, the venom required should be used and any surplus product discarded. Unused venom should not be re-lyophilized, re-dried or refrozen (in the case of liquid venom).

10.2 National reference materials

The quality of snake venoms used as a reference standard by quality control laboratories and NRAs is crucial. WHO recommends that national reference venom collections be established and that these cover each medically important snake species used in antivenom production. Such reference venoms should be prepared as described elsewhere in these Guidelines (see sections 9 and 21), and must be tested for potency at least annually to ensure they comply with the original specifications.

Owing to the large variations in venom composition even within a single species it is recommended that national reference venom collections should be established, which cover the entire interspecies variability. Regional reference materials could be used when countries within a region share a similar distribution of venomous snakes.

Establishing a collection of reference venoms ensures that the antivenoms produced will be tested against the same relevant venoms in the specific countries or regions. The characterization and maintenance of reference venom collections should be performed under oversight from NRAs and other competent agencies with technical expertise to ensure that reference venoms are produced to international reference material standards.

Venom batches may be prepared following the procedure outlined in section 9. Whatever their origin, the snakes used for these reference standards should be accurately authenticated (species, subspecies) by a qualified person and

the place of capture recorded. Genetic samples (for example, tissue and blood) should be routinely collected from all specimens for DNA analysis if questions arise regarding the validity of the identification of specimens. Photographs of individual specimens may also have value.

10.3 Characterization of venom batches

It is the responsibility of the venom producer to provide clear information pertaining to the species, the subspecies and the geographical origin of the snakes used for the production of the venoms supplied for antivenom production, quality control and preclinical studies. This information should be included in the technical dossier supporting the marketing authorization of any antivenom. In addition to the certificate which details the scientific name of the snake species (and subspecies if any), the geographical origin and the number of animals used for preparing the batch, and the date of collection of the venom as well as additional biochemical and biological information may be provided for each venom batch as evidence of consistency.

This information may include analysis of:

- biochemical characteristics of the venom;
  - protein concentration per gram,
  - scans or pictures of SDS-PAGE (in reducing and non-reducing conditions), and
  - size-exclusion or reverse-phase chromatographic profiles (for example, reverse-phase HPLC);
- enzymatic and toxicological activities of the venoms;
  - for example, LD_{50} and, depending on the particular venom, in vitro procoagulant activity, proteinase activity or phospholipase A2 activity;
- for lyophilized, vacuum-dried or desiccated venoms, analysis of residual moisture.

If the venom producer is not able to perform these determinations they can be subcontracted. Alternatively (depending on the agreement), the antivenom manufacturer can perform relevant assays to confirm compliance of venoms with specifications as part of the quality control of the raw material.

10.4 Main recommendations

- **Quality control of snake venoms is essential to provide assurance that the venoms are representative of venomous snakes inhabiting the region for which the antivenoms are manufactured.**

- **National reference venom collections should be established covering each of the medically important snake species for which antivenoms are produced.**
- **Traceability of each venom batch is important for rapid detection of any errors that might occur during the preparation process.**
- **For each venom batch, a certificate stating the scientific names of the snake species (and subspecies if any), their geographical origin and the number of animals used in collecting the batch, the date of collection of the venom, and any other relevant information, must be provided by the venom supplier to the antivenom manufacturer and also to the regulatory authority if required.**
- **Consistency (within established limits of composition and quality) of venom batches produced over time for the same venomous species of the same origin should be guaranteed. Specific tests should be performed on each venom sample, and data recorded for traceability, including: the protein concentration per g (or mg), an assessment of biochemical and biological activity, scans or pictures from SDS-PAGE (in reducing and non-reducing conditions), and/or size-exclusion or reverse-phase HPLC chromatographic profiles of the venom sample. This information has proved useful to confirm the origin and the integrity of the venom.**

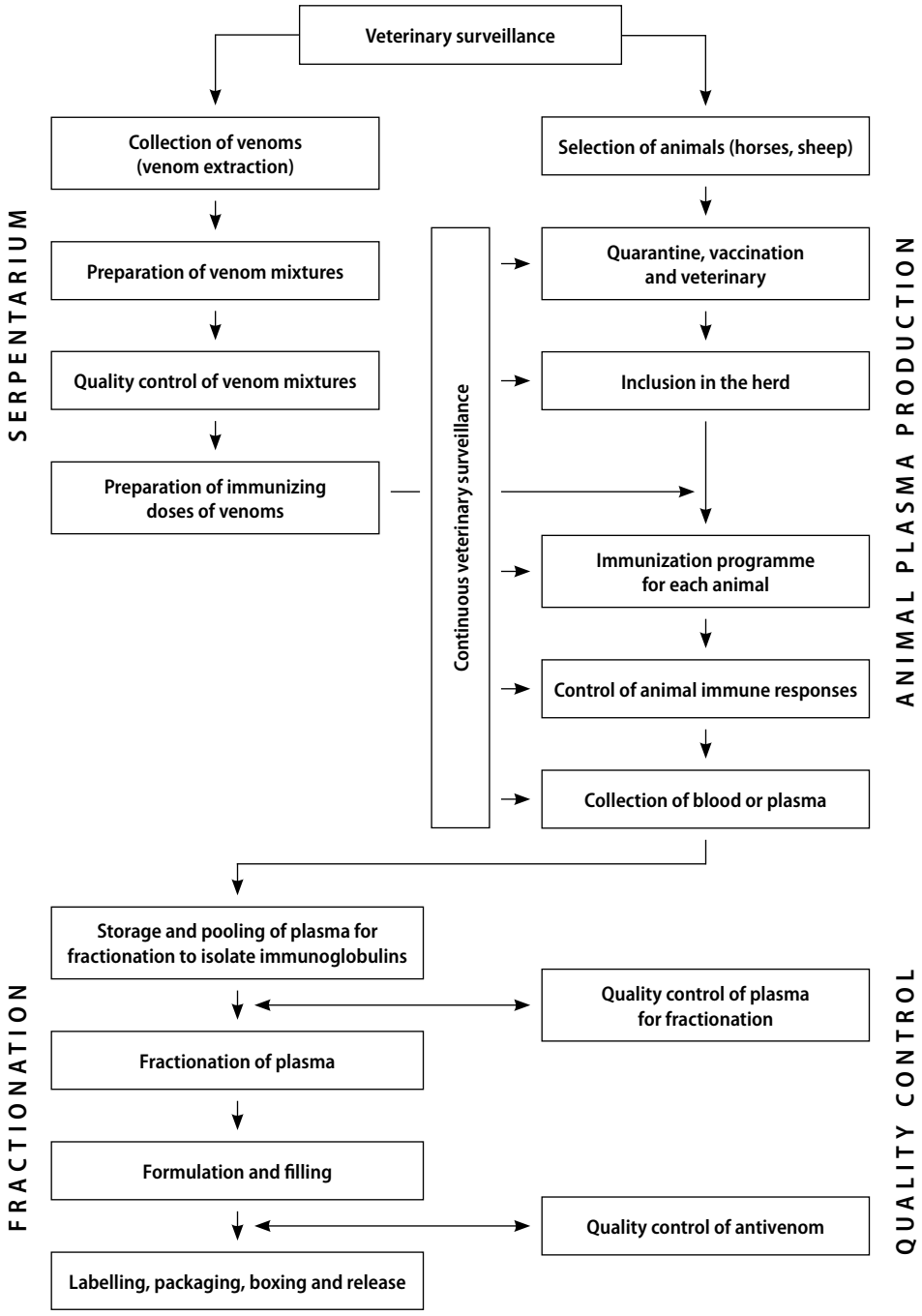
11. Overview of the production process of antivenoms

Antivenoms are obtained following a complex production process (Fig. A5.3), which involves several steps critical to their effectiveness, quality and safety (*71*). These steps are summarized below:

- Collection of representative venom pools from correctly identified individual venomous snakes which have been confirmed to be in good health. They should be representative of the snake populations (for example, males/females, adults/juveniles) and region(s) where the resulting antivenom immunoglobulins are intended to be used.
- Preparation of the venom(s) mixture(s) used for the programme of immunization of animals.
- Immunization regimens of animals (most often horses). Animals should be selected and controlled carefully, and subjected to continuous health surveillance.

- Collection of blood or plasma from the immunized animals, once the immune response to the immunizing venom mixture has yielded satisfactory antibody levels.
- Preparation of the pool of plasma for fractionation.
- Fractionation of the plasma to extract and to purify the antivenom immunoglobulins if applicable.
- Formulation of the bulk antivenom immunoglobulins and aseptic filling.
- Quality control tests, including potency assessment by in vivo assay.
- Labelling, packaging, boxing and release.
- Distribution within the region(s) where the snakes used to prepare the venoms to immunize the animals are prevalent.

Fig. A5.3
General manufacturing process of antivenoms



12. Selection and veterinary health care of animals used for production of antivenoms

12.1 Selection and quarantine period

Animals selected for antivenom production should comply with specific selection criteria relating to breed, size, age, health status and history, and should preferably be purchased from known accredited suppliers. The use of animals in the production of hyperimmune plasma should follow strict ethical standards in accordance with national and international conventions on the use and welfare of animals. Animals must be transported according to local transport standards. Before an animal is introduced into the herd used for a production programme, it should be subjected to a period of quarantine (which, in most countries, lasts from 6 to 12 weeks), depending upon the source of the animal. During the quarantine period an appropriate veterinary assessment should be performed to ensure the animal's suitability for the programme. The quarantine facility should be separate from the main animal housing facility or farm and a biosecurity plan for all animal premises is recommended. Each animal should have an individual monitoring record system created on its entry into the quarantine facility, which will remain with the animal throughout its life at the facility or farm. All activities and information on all aspects of its life, including husbandry, health, antivenom immunization, bleeding and emergency care must be recorded in this file, which should be accessible for external review.

When an animal is imported from a country or region with different ecological characteristics, a period of acclimatization to the local environment of about 3 months is needed. Each individual animal should be unambiguously identified using, for example, a microchip, branding or ear clipping.

In the case of horses and other equines, animals aged between 3 and 10 years are usually included in an immunization programme, but in some cases older animals may also be suitable as long as they exhibit a satisfactory immune response to the immunization programme. In the case of sheep, animals retired from wool production have proved capable of useful antibody production for a number of years (beyond the age of 10 years). No particular breed is preferred, but in general large horses or sheep are chosen because they yield larger individual volumes of blood.

12.2 Veterinary care, monitoring and vaccinations

The veterinary examination will include a complete physical examination and blood tests including serological testing for the most prevalent infectious diseases for that type of animal in that particular geographical location (for example, equine infectious anaemia).

Depending upon the local epidemiological situation, animals should be vaccinated against tetanus and, possibly other endemic diseases, such as rabies, equine influenza, anthrax, brucellosis, glanders, African horse sickness and equine encephalitides. Animals should go through a treatment programme to eliminate gut helminths and other locally prevalent parasites. All vaccinations and health information should be recorded on the animal's individual record.

Staff in contact with the animals should be vaccinated against tetanus and rabies.

12.3 Animal health and welfare after inclusion in the herd

After the quarantine period, if the animal is in good health according to a veterinary examination and blood parameters and body condition score, and the results of relevant serological tests are negative, the animal may be incorporated into the herd of animals used for immunization.

An individual record should be kept for each animal being used in an immunization programme for antivenom production. In addition to surveillance by a veterinary professional, the staff in charge of the animals should be well trained, and the operations related to animal care, emergency care and use should be clearly specified in the standard operating procedure (SOP).

Throughout the time an animal is used for immunization aimed at antivenom production, careful veterinary monitoring should be maintained, including continued vaccination regimes, and the performance of regular clinical examinations, together with clinical laboratory tests such as packed cell volume, haemogram, clotting tests and other tests associated with the possible clinical effects of venoms (*72*) and of successive large-volume blood collection (*73*). Possible anaemia, resulting from excessive volume or frequency of bleeding (when red blood cells are not re-infused into the animals after the whole blood bleeding session) or from the deleterious action of venoms should also be tested for.

The immune response against venom components should, when feasible, be followed throughout the immunization schedule, in order to detect when animals reach an acceptable antivenom titre. However, the monitoring of the immune response can be done on a pool of sera from various animals. This response may be followed by in vivo potency assays of neutralization of lethality or by in vitro tests, such as enzyme immunoassays (EIAs) (provided that a correlation has been demonstrated between these tests and the in vivo potency tests).

Whenever an animal develops any manifestation of sickness, it must be temporarily withdrawn from the immunization programme to allow it to receive appropriate veterinary examination and treatment. If the disease is controlled, the animal may return to the immunization programme after a suitable length

of time, usually 4 weeks. If an animal is receiving any type of antibiotic or drug, it should be withdrawn from the immunization programme for a period that would depend on the elimination kinetics of the particular drug(s) concerned. In the case of vaccination, this withdrawal period should not be shorter than 1 month.[6] Any blood, plasma or serum obtained from the animal during the incubation period of any contracted disease should be excluded from use for the production of antivenoms. Animals should have appropriate physical exercise and routine husbandry (hoof care, teeth rasping etc.). Their feed should originate from a controlled source and should be free of ruminant-derived material. Ideally, the diet should include both hay and grass, or alternative plant material, and concentrated food preparations containing vitamins including folic acid, iron and other mineral supplements. The routine quality control of the food and water is recommended, in order to assure a consistent composition and an adequate level of nutrients.

As a consequence of immunization with venoms (see section 13) a common problem in antivenom-producing animals is the development of local ulcers or abscesses (sterile and infected) at sites of venom injection. This is a particular problem when necrotic venoms and FCA are used. All injections should be given under aseptic conditions and administered subcutaneously. There should be a limit to the total volume and dose of venom injected at a single site. Infected or ulcerated areas should be treated appropriately. Abscesses should be lanced and drained and the affected skin site should not be used again. In the event of the death of an animal being used for antivenom production, a careful analysis of the causes of death should be performed, including, when necessary, the performance of a necropsy and histopathology. All deaths should be recorded together with the necropsy report and made available for external review.

Some animals show declining titres of specific venom antibodies over time, despite rest or increasing doses of immunizing venoms. Such animals should be retired from the immunization programme. In agreement with the principles of GMP and to avoid impact on the composition and consistency of the antivenom produced, it is, in principle, not considered good practice to move animals from a given venom immunization programme to another one. If, however, the animal has been used in the preparation of a monospecific antivenom that is included in a polyspecific preparation, or if it was used for the production of other animal-derived antisera (for example, anti-rabies, anti-tetanus or anti-botulism), moving it to another programme may be acceptable.

When an animal is withdrawn from the herd, it may be kept on the horse farm. If it is sold, continued good care should be ensured.

[6] In some areas, legislation stipulates that animals used for production of plasma cannot be treated with penicillin or streptomycin.

12.4 Main recommendations

- **A thorough biosecurity plan should be developed and implemented for each farm and facility.**
- **All staff working with the animals should be trained and qualified to care for them. Staff training records and history should be available for review.**
- **An emergency care protocol is essential especially during procedures – for example, during sensitization to venoms, blood collection and post-plasmapheresis. Adverse events must be reported and tracked appropriately.**
- **Animals intended for antivenom production programmes should be identified to ensure full traceability and health monitoring.**
- **Animals should go through a quarantine period of 6–12 weeks during which they are submitted to veterinary scrutiny and are vaccinated against specific diseases and treated for internal and external parasites.**
- **Following the quarantine period, animals may be introduced into the immunization programme. Animals should be appropriately housed, fed and managed according to best practice in veterinary, animal welfare and ethical standards.**
- **During immunization, the clinical status of each animal must be followed by a veterinarian through clinical and laboratory assessments which are recorded on the animals' records. If an animal develops clinical signs of disease, it should be temporarily separated from the immunization programme to receive appropriate care and treatment. Particular care must be paid to local lesions that develop at the site of venom injections and to development of anaemia.**
- **The immune response of each animal to venoms should, when possible, be monitored during the immunization schedule (alternatively, the antivenom titres can be monitored indirectly by testing the plasma pool).**
- **An animal receiving an antibiotic or drug should be withdrawn from the immunization programme for a period depending on the elimination kinetics of the drug concerned. In the case of vaccination, this withdrawal period should not be shorter than 1 month.**

13. Immunization regimens and use of adjuvant

One of the most crucial steps in antivenom production involves the immunization of animals with venom(s) to produce a long-lasting and high-titre antibody response against the lethal and other deleterious effects of the immunogenic toxins. To achieve this goal, the following considerations are important:

- Venom(s) used should be prepared as described in section 9, and should be in an optimal condition for inducing specific and neutralizing antibodies.
- Immunogen and the immunization regimens used should not seriously affect the health of the animal.
- Preparation of immunogens and the immunization protocol should be technically simple and economical and use a minimal amount of venom. The procedures followed must be included in a protocol and their performance must be documented.

The antivenom manufacturer is responsible for defining the appropriate immunization programme (choice of doses, selection of adjuvants, sites of immunization, and bleeding schedule) able to generate the best immune response and plasma production, while also ensuring optimal animal care. GMP principles should be applied in the preparation of the immunizing doses as well as in the immunization process.

13.1 Animals used in antivenom production

Numerous animal species have been used on various scales in antivenom production (horse, sheep, donkey, goat and rabbit) or for experimental purposes (camel, llama, dog and hen) (*74*, *75*). However, the production of large volumes of antivenom from large animals such as equines is an advantage compared to the smaller species. The selection of the animal species should be based on several considerations, such as locally prevalent diseases, availability in the region, adaptation to the local environment, and cost of maintenance. The information in these Guidelines refers mostly to horse-derived immunoglobulins.

The horse is the animal of choice for commercial antivenom production. Horses are docile, thrive in most climates and yield a large volume of plasma. Antivenoms made from horse plasma have proven over time to have a satisfactory safety and efficacy profile (*3*). Sheep have also been used as an alternative source for antivenom production because they are cheaper, easier to raise, can better tolerate oil-based adjuvant than horses, and their antibodies may be useful in patients who are hypersensitive to equine proteins (*75*, *76*). However, increasing concern about prion diseases may limit the use of sheep

for commercial antivenom production. Larger animals are preferable to smaller ones because of their greater blood volume, but breed and age are less important. Any animals used should be under veterinary supervision (see section 12). When sheep or goats are to be used, manufacturers should comply with regulations to minimize the risk of transmissible spongiform encephalopathies (TSEs) to humans, such as the *WHO Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies* (*77*).

13.2 Venoms used for immunization

Venoms used as immunogens in antivenom production are chosen based on criteria discussed in section 9. Priority should be given to venoms from snakes responsible for frequent and severe envenomings. The quality, quantity and biological variation of venoms are important considerations (see sections 9 and 10).

13.3 Preparation of venom doses

Venom doses used for the immunization of animals should be prepared carefully in a clean environment, maintained according to an established, scheduled and documented cleaning regime. All venom manipulations should be performed using aseptic techniques under a hood; for highly toxic venoms, a cytotoxic cabinet may be used. Batch process records should be completed for each dose preparation session. The venom batches used and the animals to be immunized should be recorded and the containers in which the venom is dissolved should be appropriately identified. Ideally, the calculations and operations related to the dose of venom to be used, as well as dilutions, require verification by a second person to ensure accuracy and to prevent errors that may lead to animals receiving overdoses.

Venoms, when freeze-dried, are highly hygroscopic and allergenic, thus care should be taken when manipulating them. When taken out of the refrigerator or freezer, the venom should be allowed to warm up to room temperature before the bottle is opened. Otherwise, condensation may occur causing inaccuracy in weighing and, more seriously, proteolytic degradation of the venom proteins by venom enzymes. Venom should be dissolved in distilled water or buffer, but care should be taken not to shake the solution too vigorously since excessive foaming may cause protein denaturation.

The solvents used to dissolve venoms should be sterile and should be used before the established expiry dates. A stock solution of each venom should be prepared separately, rather than being mixed with other venoms. This is to allow flexibility of dosage and to avoid proteolytic degradation by one venom component of other venom proteins. Venom solutions can be sterile filtered (where this is known not to affect the potency of the preparation), aliquoted

and labelled. It is recommended that venoms used for immunization be freshly prepared at the time of use. The storage conditions of venom solutions before immunization must be validated by properly conducted stability tests.

All the equipment used for venom storage (freezers and refrigerators) and preparation (for example, balances) should be calibrated and validated for their intended purpose. Balances should be calibrated at least annually and calibration should be checked daily. Where possible, laboratory items used in venom preparation, that is, pipettes, syringes and other such items should be pre-sterilized, single-use, disposable items. The siliconization of venom solution containers may be considered to avoid the adherence of venom components to the surfaces of containers. Venom solutions/suspensions must be safely transported to the facilities where animals are going to be injected and the venom solutions/suspensions should be kept cold at about 2–8 °C.

Care should be taken to avoid accidents that may result in envenoming of the personnel preparing the venom solutions. Protective equipment (for example, eyewear, gloves and gowns) should be worn. Procedures for cleaning up broken glass or plastic containers that have held venom should be established and the personnel should be trained to follow them.

13.4 Detoxification of venom

Some snake venoms can cause local and/or systemic toxicity when injected into naive horses at the beginning of an immunization course. Various physical or chemical means have been adopted to decrease venom toxicity, for example, treatment with aldehydes (formaldehyde or glutaraldehyde), hypochlorite, ultraviolet or gamma radiation, and heat, among others. However, in most cases, not only the toxic sites, but also the antigenic sites of the toxins are destroyed after these treatments (*78*). For example, when glutaraldehyde is used, the protein polymerization is often extensive and is difficult to control and reproduce. Thus, although the detoxified toxin (toxoid or venoid) induces vigorous antibody response, the antibodies usually fail to neutralize the native toxin. In fact, no detoxification is usually necessary if inoculation is made with a small dose of venom that is well emulsified in an adjuvant such as FCA or FIA. Furthermore, traces of chemicals, especially aldehyde, can have deleterious effects on animals' vital organs, for example, the liver.

13.5 Immunological adjuvants

Various types of immunological adjuvants have been tested, for example, FCA and FIA, aluminium salts (hydroxide and phosphate), bentonite and liposomes (*79*). The choice of adjuvant is determined by its effectiveness, side-effects, ease of preparation (especially on a large scale) and cost. It may vary depending upon the type of venoms and following manufacturers' experience. FIA contains

mineral oil and an emulsifier. FCA, which contains mineral oil, an emulsifier and inactivated *Mycobacterium tuberculosis*, has been shown in experimental animals to be one of the most potent adjuvants known. However, horses are quite sensitive to FCA which tends to cause granuloma formation. For this reason, some producers prefer to use other adjuvants. It is recommended that when using FCA and FIA, they be utilized only at the beginning of the immunization schedule, and not during the rest of the immunization, nor during booster injections of venom; this significantly reduces the formation of granulomas in horses.

It has been noted that the granuloma caused by FCA is caused by the injection of a large volume (5–10 mL) of the emulsified immunogen at one or two sites. The large granuloma formed usually ruptures, resulting in a large infected wound. If the emulsified immunogen is injected subcutaneously in small volumes (50–200 µL/site) at multiple sites, granuloma formation may be avoided. Manufacturers are also encouraged to adopt an innovative approach with regard to adjuvants used for antivenom production, and should strive to replace FCA and FIA with new compounds of low toxicity and high adjuvant effect. The advances in the vaccine field concerning new adjuvants should be transferred to the antivenom field; for example, the use of microbial-derived products of low toxicity or of Toll-like receptor 4 (TLR4) ligand-based adjuvants (*80*).

13.6 Preparation of immunogen in adjuvants

To minimize infection at the immunization sites, all procedures should be carried out under aseptic conditions. Venom solutions are prepared in distilled water or phosphate-buffered saline solution and filtered through a 0.22 µm membrane. The venom solution is then mixed and/or emulsified with adjuvant, according to the instructions of the supplier. An example for the preparation of venom immunogen in FCA, FIA and aluminium salts is described in Box A5.1. To facilitate the injections, the immunogen suspension is filled in tuberculin syringes (Fig. A5.4).

13.7 Immunization of animals

The areas to be immunized should be thoroughly scrubbed with a disinfectant, shaved and rubbed with 70% ethanol before venom immunogen injection.

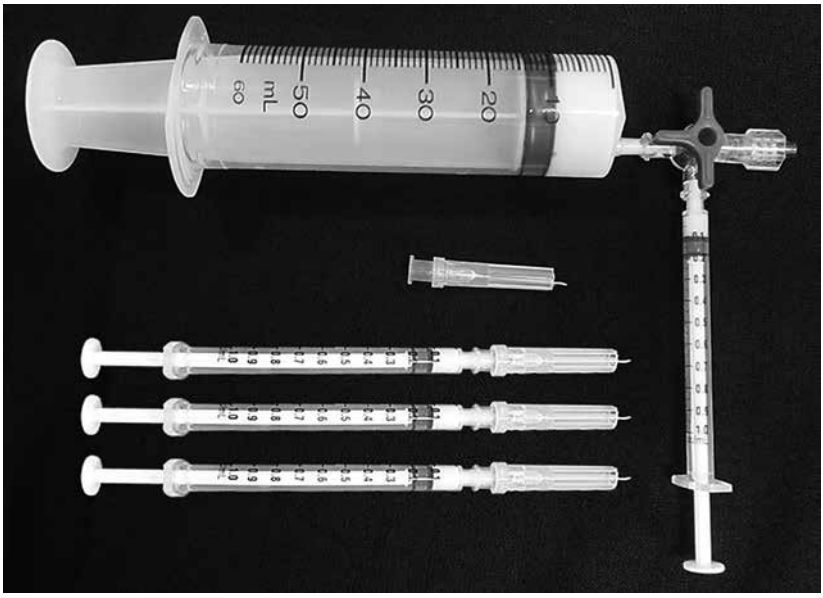
In general, the sites of immunization (Fig. A5.5) should be in areas close to major lymph nodes, preferably on the animal's neck and back. The route of injection should be subcutaneous so as to recruit a large number of antigen-presenting cells resulting in a high antibody response. Some procedures call for a small volume of injection at each site (50–200 µL) so that the total surface area of the immunogen droplets is maximized, enhancing the interaction with the antigen-presenting cells and the immune response (*81*, *82*). An example

of immunization of a horse using venom emulsified in FCA is described in Box A5.2.

Other immunization protocols, using larger amounts of venoms devoid of local-tissue-damaging activity (such as those of some elapids) and/or adjuvants other than FCA may be used with satisfactory results, as long as the schedule does not compromise the health of the animals. In situations where the main toxins of a given venom have a low molecular mass and would not induce a sufficient immune response if injected together with the other venom components, isolating such toxins using mild chromatographic procedures or ultrafiltration can be useful. Such isolated fractions can then be used for immunization.

Fig. A5.4

Tuberculin syringes are filled with immunogen suspension and used for the subcutaneous injection of the horse



Box A5.1

Example of preparation of venom immunogen in FCA, FIA and aluminium salts

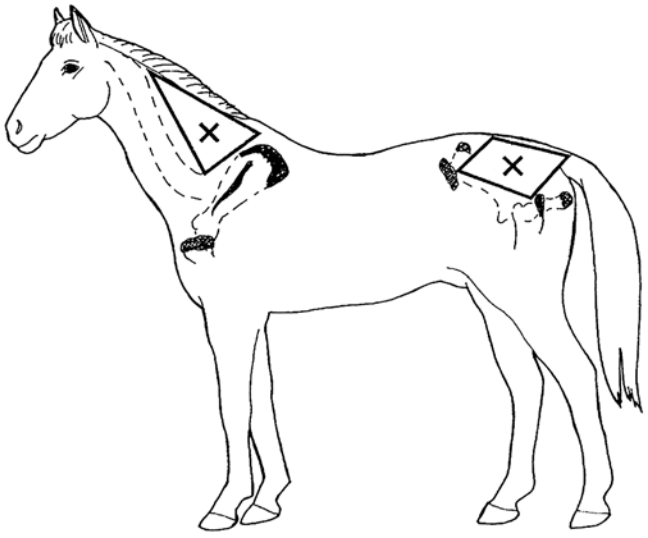
Since FCA can cause severe irritation, precautions should be taken to avoid contact with the eyes, and protective eyewear and gloves are recommended. The vial containing FCA is shaken to disperse the insoluble *Mycobacterium tuberculosis*. The venom solution is mixed in a stainless steel container with an equal volume of FCA at 4 ºC.

Box A5.1 *continued*

The emulsification is achieved by vigorous blending in a high-speed blender at a speed of approximately 3000 rpm for 15 minutes. The container is put in ice-water to dissipate the heat generated. The resultant emulsion should be quite thick and remain stable when dropped on the surface of cold water. The highly viscous emulsion is then transferred into a sterile 50 ml syringe with the plunger removed. The plunger is then put into the syringe to expel any air pocket inside. By means of a three-way stopcock, the emulsion from the 50 ml syringe is then transferred into tuberculin syringes to give a volume of 0.1–0.2 ml/syringe. After the tuberculin syringe is fitted with a 38 mm no. 21 gauge disposable needle, the needle cover with its end cut off is attached so that only 2–3 mm of the needle tip is exposed and penetrates the horse's skin (Fig. A5.5). With each filled tuberculin syringe, immunization at a particular site can be performed by injection and expulsion of the immunogen almost simultaneously in one single step. This immunization procedure makes multiple subcutaneous injections with small immunogen volume easier, faster and requires minimal restraint of the horse.

Immunogen in FIA is prepared by a process similar to that described above except that FIA is used in place of FCA. Both the FCA and FIA emulsified immunogens may, if necessary, be stored at 4 °C, preferably for a maximum of 2 weeks, but re-emulsification is necessary before their injection. When the immunogen is prepared in aluminium hydroxide ($Al(OH)_3$) or aluminium phosphate ($Al(PO)_4$), a sterile venom solution and a suspension of aluminium salts are mixed in a ratio of 1:3 (v/v) and homogenized. When using other adjuvants, the preparation of the solution or emulsion should follow the manufacturer's instructions for that type of adjuvant.

Fig. A5.5
Recommended areas of immunization in horses



Box A5.2
Example of immunization of horses using FCA, FIA and aluminium salts

The primary immunization could be done with venom(s) mixed with FCA as described in Box A5.1. The initial dose of each venom could be as low as 1–4 mg/horse with a total combined volume of injection of about 2 ml. The immunogen is filled in several 1 ml tuberculin syringes with 21G needles as described in Box A5.1 and Fig. A4.5. Subcutaneous injections of 100–200 µL of immunogen are made at each site, up to as many as 8–12 sites, although some producers may use only 3–4 injection sites. The neck of the horse, supplied with extensive lymphatic vessels and large lymph nodes, is a preferred area for immunization. If inoculation is made on the lateral sides of the neck, the animal tends to rub itself causing skin blisters. Thus, injections should be made to the upper (dorsal) part of the neck, close to the mane. About 4–6 injections can be made at each side of the neck. If injection in the rump is possible, 1–2 injections can be made in the area between the outer hip bone and the top of the thigh bone. The scratching of injected sites by animals can be partially alleviated by massaging the injection site after venom injection to disperse the dose material.

Immunization using FCA is usually done only once; in most cases, repeated use of this adjuvant can cause serious reactions which affect the horse's health. After 2 weeks, the horses should receive a booster injection with the same venom(s) well emulsified in FIA. Similar volumes and areas of injection to those described above can be used. Subsequent booster immunizations at 2-week intervals can be administered, with higher doses (5–10 mg) of venom(s) in saline or mixed with aluminium salts or any other suitable adjuvant. In this case, subcutaneous injections of 1 ml of immunogen at each site in a total of 4 sites are recommended. Blood (10–20 ml) should be drawn before each immunization. Serum or plasma is prepared and EIA titres and/or lethality potency are determined. When the EIA titres reach a plateau, usually about 8–10 weeks after the primary immunization, an in vivo potency assay may be performed to confirm that the horse could be bled. After bleeding for antivenom production, the horses are allowed 4–8 weeks rest, depending on their physical condition. After the rest period, a new round of immunization can be performed as described above, but without the use of FCA.

13.8 Traceability of the immunization process

The traceability of the immunization process is critical for the quality control of the antivenoms produced and the steps to ensure traceability should be performed very accurately. Each immunized animal should be identified by its code number (see section 12) and the details of each immunization step should be recorded precisely. The details to be recorded include:

- date of immunization;
- batch(es) of venom(s) used with its (their) reference number(s) (see section 10);
- venom dose(s);

- adjuvant and/or salt used;
- names of the veterinary and supporting staff in charge of the immunization;
- occurrence of reaction and/or sickness.

The antivenom titre of the immunized animals should be monitored throughout the immunization procedure either in vitro, using EIA, during the immunization phase, or in vivo, by neutralization potency assays of lethality when the immunization plateau is reached or before each blood collection.

Each plasma batch should be assigned a unique reference number (for example, a barcode), which should allow complete traceability to the donor animal. Information (such as the date of collection, the unique identification number of the immunized donor animal, and the reference number of the venom(s) used for immunization) should be recorded to allow traceability to all venoms. Computer-based databases are very useful for properly recording these data, which are crucial for the traceability of the antivenoms produced. Standard procedures should be used to protect the integrity of data stored on a computer, including regular, frequent backup, protection against unauthorized access and storage of backup copies securely off-site.

13.9 Main recommendations

- **Venom solutions should be prepared in such a way as to minimize proteolytic digestion and denaturation of the venom proteins. Venom solution should be prepared under aseptic conditions to avoid infection at the injection sites.**
- **The type of adjuvant used is selected on the basis of its effectiveness, side-effects, ease of preparation and cost.**
- **Primary immunization should be done by subcutaneous injections of small volumes at multiple sites close to the animal's lymphatic system to favour the recruitment of antigen-presenting cells and involvement of anatomically different groups of lymph nodes for antibody production.**
- **Subsequent booster injections can be made using venom immunogen doses, at volumes and intervals depending on the type of adjuvant used, until the antivenom titre reaches a plateau or a pre-established minimum accepted titre.**
- **After collection of blood for antivenom production, animals should have a resting period of 4–8 weeks. After this, a new round of immunization can be performed as above without the use of FCA.**

- **All steps in the immunization of the donor animal, as well as the collection of blood or plasma, should be traceable.**

14. Collection and control of animal plasma for fractionation

Historically, serum separated from the blood of hyperimmunized horses was the basis of "antivenin serum therapy", but today plasma is used, almost exclusively, as the starting material and undergoes a fractionation process for the separation of purified antivenoms. Thus "antivenom immunoglobulins" is the preferred term, rather than "anti-snake-bite serum" or "antiserum", which are imprecise and confusing terms that refer to a crude therapeutic preparation.

Plasma as a starting material is preferred to serum largely because red blood cells can be returned to the animal, thus preventing anaemia and hypovolaemia in the donor animal and allowing more frequent bleeding. Some laboratories have found that using plasma enables higher recovery of antibodies per donation and it is less contaminated with haemoglobin (Hb) than serum. Separation of plasma from anticoagulated blood is much faster than separation of serum from clotted blood. Plasma for fractionation can be obtained either from the collection of whole blood or by the apheresis procedure.

14.1 Health control of the animal prior to and during bleeding sessions

When an immunized animal has developed an antivenom antibody titre that meets the necessary specifications it can be bled. The animal should be in a satisfactory clinical condition and blood parameters and biochemistry need to be within the normal range for the animal type and breed. Before bleeding is performed, the animals should be evaluated by a veterinarian or other qualified person and declared healthy. Individual blood chemistry parameters – packed cell volume (PCV); Hb and total plasma protein (TPP) – must be within specified parameters. Animals showing evidence of clinical deterioration, such as weight loss, altered horse body condition score, a drop in Hb or serum protein concentration below a critical predefined value for the animal type and breed, or evidence of infection, should not be bled. It is recommended that animals to be bled have no contact with potentially infectious animals. Human beings can be a potential source of fomite infection in horses and therefore a biosecurity plan is essential.

14.2 Premises for blood or plasma collection

The bleeding of animals should be performed in designated rooms or areas dedicated to this activity and equipped with appropriate restraining devices. Some producers may design the bleeding rooms so that they can be closed, if needed, during the bleeding sessions, but this is not general practice. The rooms or areas should be thoroughly washed and cleaned before and after each bleeding session and their design should facilitate such cleaning procedures, which should be clearly established. The room or area should be inspected before the confinement of the animal. Animals need to be made as safe and comfortable as possible, in a quiet environment, during bleeding to minimize the chance of injury to the animal or its handlers. Individual animals should be confined in circumstances that reduce the potential for stress as much as possible. It is recommended that these rooms allow the simultaneous bleeding of several animals to reduce the time required for this operation as well as the stress.

To avoid bacterial and fungal contamination, animals should be cleaned and injection sites and jugular catheter sites clipped in a separate room before bleeding. Humidity control of the surrounding bleeding area should be ensured.

14.3 Blood or plasma collection session

Animals are bled by venepuncture from the external jugular vein. The area surrounding the venepuncture site should be clipped before bleeding and thoroughly cleaned and disinfected, using a disinfectant that has not reached the end of its recommended shelf-life, and, depending on the type of disinfectant, it should be allowed to dry. The disinfected area should not be touched or palpated before the needle has been inserted.

Before venepuncture, all containers and tubing should be inspected for defects (for example, abnormal moisture or discoloration as these may suggest a defect). There should be means to determine the volume of blood or plasma collected (such as a weighing machine).

When using disposable plastic bags to collect blood (which may take about one hour) it is recommended that the blood should be gently and continuously mixed to ensure a homogeneous distribution of the anticoagulant and avoid formation of clots.

Horses should be weighed before bleeding, if possible, and their weight recorded on their record. The clinical condition of the animals being bled should be closely monitored at the time of bleeding and during the days that follow, and bleeding should be suspended in the event of any adverse effect on the animal. If an animal shows signs of distress during the operation, the collection procedure should be terminated. In addition, animals should be kept under observation for at least 1 hour after the bleeding to allow any evidence of

physical alterations to be detected. Horses can be fed during blood collection depending on the horse crush set-up.

After bleeding for antivenom production and depending on whether red blood cells are re-transfused, the horses should not be re-bled for 4–8 weeks. Horses should have moderate (5 out of 9) body condition score and normal range haemogram (PCV, TPP and Hb) and be normal on clinical examination by the antivenom production veterinarian.

14.4 Labelling and identification

The identity of the animal should be recorded immediately before venepuncture. Labels on all bottles or bags of blood or plasma should be marked with the animal's unique identification number. The label should be waterproof and heat resistant, and contain the following information: specificity of antivenom, plasma unit number and date of collection.

A document to register all steps of the production of the plasma lot should be maintained to guarantee traceability of the process.

14.4.1 Collection and storage of whole blood

14.4.1.1 Collection

The volume of blood to be obtained depends on the species and size of the immunized animal. It is recommended that approximately 13–15 mL of blood per kilogram body weight are collected in one bleeding session, or 1.5 to 2% of the weight of the animal. For sheep, 0.5 L is a typical yield, whereas for horses, the volume of blood may range between 3 and 6 L, depending on the size of the animal. The use of automated plasmapheresis may enable larger volumes of plasma to be collected, and has benefits for animal health and plasma quality. Manufacturers are encouraged to evaluate and implement automated plasmapheresis subject to approval from local regulators and in accordance with local regulations and standards.

Blood is collected, ideally, in disposable plastic bags containing sterile citrate anticoagulant or other preparations containing citrate phosphate dextrose solution (CPD), to prolong the durability of red blood cells. Usually, the volume ratio of anticoagulant to blood is 1:9 to 1:15, depending on the anticoagulant. Use of double plastic bags containing anticoagulant is recommended to avoid bacterial contamination and for ease of use. When plastic bags are not available, disposable polypropylene plastic bottles, or sterilized glass bottles containing anticoagulant may be considered.

While the bleeding is taking place, a constant flow of blood should be ensured. Blood should be gently and continuously mixed with the anticoagulant solution to ensure a homogeneous distribution of the anticoagulant, to avoid the risks of activation of the coagulation cascade and, therefore, avoid the formation

of clots. The duration of a bleeding session per animal is usually between 30 and 45 minutes depending upon the weight of the animal and the total volume collected. Care should be taken to avoid contamination of the blood by exposing the needle to contaminated surfaces. It is recommended to seal or occlude the device before removing the needle from the animal.

14.4.1.2 Storage

The bags or bottles in which the whole blood has been collected should be appropriately cleaned and sanitized on their external surfaces. They should be put into a refrigerated room (2–8 °C) for the plasma and blood cell separation procedure. They should be stored for no more than 24 hours before the reinfusion of the red cells, unless CPD is used. In this case, blood cells may be stored for up to 72 hours. Alternatively, aseptically collected blood can be stored for a maximum of 7 hours at 20–25 °C to allow for sedimentation. Under such circumstances, great care should be taken to avoid bacterial contamination.

14.4.1.3 Separation of plasma from whole blood

Hyperimmune plasma should be separated from blood cells under aseptic conditions and should be transferred into sterile containers (plastic bags, bottles or stainless steel containers). A designated room, designed to allow proper cleaning and sanitization, should be used for separation. When bottles are used, separation of plasma from blood cells should be performed in a laminar flow cabinet located in a room separated from the plasma fractionation area.

14.4.1.4 Reinfusion of the red blood cells

Reinfusion of the red blood cells after whole blood collection is recommended. Blood cells, most specifically red blood cells, should be separated from plasma by validated centrifugation or sedimentation procedures. Red blood cell reinfusion should take place within 24 hours after blood collection (or 72 hours if CPD anticoagulant is used), and after being suspended in sterile saline solution at room temperature for 1 hour (or 32–37 °C for less than 1 hour) prior to infusion. This procedure in which whole blood is collected and red blood cells are re-infused into the animal is commonly referred to as "manual apheresis".

14.4.2 Plasma collection by automatic apheresis and storage

14.4.2.1 Plasma collection

In some laboratories, plasmapheresis machines are used to perform automatic plasma collection. This has proved a useful investment in some facilities; it ensures that the donor animal does not become hypovolaemic, increases plasma yield and purity, and reduces the risks of handling errors, in particular

during reinfusion of the red blood cells to the donor. Plasma from automatic apheresis tends to be less contaminated by blood cells (red blood cells, leukocytes and platelets). In the experience of some laboratories the plasma is easier to fractionate, as the filtration steps, in particular, are more readily performed, resulting in higher yields.

In such procedures, whole blood is collected from the animal, mixed with anticoagulant, and passed through an automated cell separator. The plasma is separated from the cellular components of the blood, which are returned to the animal in a series of collection/separation and return cycles. The plasma is separated from the red blood cells by centrifugation or filtration, or a combination of the two. The operational parameters of the plasmapheresis equipment are provided by the manufacturers of the equipment. In general, the anticoagulant is delivered at a rate yielding a specified ratio of anticoagulant to blood. The anticoagulant solutions used include AB16 (35.6 g sodium citrate, 12.6 g citric acid monohydrate, 51.0 g glucose monohydrate per litre using water for injection) and anticoagulant citrate dextrose formula A (ACDA) (22.0 g sodium citrate, 8.0 g citric acid, 24.5 g dextrose monohydrate, per litre using water for injection). The number of collection/separation and return cycles for each donor animal depends on the total volume of plasma that is to be harvested. For horses, the average volume of plasma collected may be about 6 litres per session. The number of cycles ranges from 10 to 20 depending upon the haematocrit of the horses. The collection process lasts for 1–4 hours. The apheresis equipment and apheresis procedures should be validated, maintained and serviced. Machine plasmapheresis can take several hours and animals can be fed during the operation.

14.4.2.2 Plasma storage

Bags or bottles containing apheresis plasma should be stored in a refrigerated room (2–8 °C) in the dark until the fractionation process starts. Individual or pooled plasma should be stored at 2–8 °C in a cold room dedicated for this purpose. This refrigerated storage room should be designed to allow proper cleaning and sanitization. To prevent microbial contamination of plasma, preservatives (phenol or cresols[7]) can be added at a dose of less than 3 g/L at this stage and kept during storage of plasma. Care should be taken to dilute the phenol or cresols with water or saline solution before they are added to plasma with gentle stirring, to avoid denaturation of plasma proteins. The transportation of containers or bottles containing pooled plasma within the production facility or between facilities should be performed in such a way that contamination is

[7] In these Guidelines cresol isomers are referred to as "cresols".

avoided and the cold chain is maintained. To avoid the risk of contamination, it is recommended that individual or pooled plasma is not stored for too long before fractionation, that is, the plasma should be fractionated as soon as possible after pooling.

If plasma is stored for prolonged periods, the storage time and conditions should be validated to ensure that there is no detrimental impact on the quality of the plasma material, on the fractionation process, or on the quality, efficacy and stability of the antivenoms.

Manufacturers of human plasma have found that plasma can be stored frozen at −20 °C or colder for 2 years without addition of a preservative, and with no observed detrimental effects on the fractionated plasma products.

14.5 Pooling

Plasma from individual animals should be pooled into sterile and sanitized containers before fractionation. For traceability purposes each plasma pool should be identified with a unique number. The number of plasma units collected from individual animals and used in the pool should be recorded. Before the large pool of plasma is prepared, it is recommended to prepare a small-volume pool and to test it for microbial contamination. If there is no contamination, the large pool can be prepared. If microbial contamination is detected, the plasma from the individual animals should be checked, and the contaminated plasma should be discarded to ensure that the pool is prepared with plasma free of microbial contamination. It may also be advisable to test small pools using a cytotoxicity assay, which can reveal the presence of unanticipated viruses or toxins (for example, following the US Code of Federal Regulations, 9 CFR 113.53 "Requirements for ingredients of animal origin used for production of biologics").[8]

Such pooling should be performed in an environment suitable to prevent microbial contamination, for example, classified areas (class D (*83*)) and pools should be adequately identified. The room should be designed to allow for appropriate cleaning and sanitization of all surfaces. Individual or pooled plasma should be stored at 2–8 °C in a room dedicated for this purpose. To ensure the prevention of microbial contamination of plasma, follow the recommendation given in section 14.4.2.2.

[8] https://www.gpo.gov/fdsys/search/pagedetails.action?packageId=CFR-2005-title9-vol1&granuleId=CFR-2005-title9-vol1-sec113-53&bread=true&collectionCode=CFR&browsePath=Title+9%2FChapter+I%2FSubchapter+E%2FPart+113%2FSubjgrp%2FSection+113.53&collapse=true&fromBrowse=true, accessed 24 April 2017.

14.6 Control of plasma prior to fractionation

Before fractionation, pools of plasma should be checked for macroscopically evident precipitates, gross haemolysis and bacterial contamination (bioburden assay). The neutralizing potency of the starting plasma should be ensured so that the resulting antivenoms will be within potency specifications. Additional checks may include, when relevant, a test for pyrogenic substances and total protein content.

Plasma pools should be discarded if the bioburden exceeds a defined limit stipulated in the marketing dossier or if the neutralizing potency is below a minimum limit established by the producer. Cloudy plasma, below this defined bioburden limit, may still be used for fractionation provided the fractionation process and product quality has been proven not to be impaired. Grossly haemolysed plasma should not be used for fractionation. Cloudy plasma may also reflect the increased level of chylomicrons in the animal plasma; therefore it is recommended to fast the animal before bleeding for a few hours (for example, 8 hours).

14.7 Main recommendations

- **When animals have developed an adequate immune response against venoms, and if they are in good health, they can be bled for antivenom production. Bleeding should be performed in enclosed rooms which should be kept scrupulously clean. Traceability of the donations should be ensured.**
- **Plasma is preferred to serum as a source material. Animals should be bled from the external jugular vein. Plasma can be obtained either from whole blood or by automated plasmapheresis and using approved anticoagulants. Blood or plasma should ideally be collected into closed plastic bags. When this is not possible, glass or plastic bottles can be used, if they can be readily cleaned and sterilized.**
- **Plasmapheresis is recommended using either automatic or manual procedures. When manual apheresis is used, blood cells should be sedimented, separated from the plasma, re-suspended in saline solution and returned to the animals within 24 to 72 hours. Plasma separation should be performed in a designated room with a controlled environment.**
- **Plasma containers should be thoroughly cleaned on their external surfaces, adequately identified and stored in refrigerated rooms until further fractionation.**

- **Plasma should be checked prior to fractionation to establish compliance with relevant acceptance criteria for fractionation, in particular the neutralizing potency and lack of bacterial contamination.**
- **Special attention should be paid to ensuring traceability between individual animal donors and the plasma pool.**
- **A certificate from a veterinarian or other qualified person should be issued stating that the donor animals were checked periodically to ensure that they were in good health at the time of plasma collection and during the follow-up observation period.**

15. Purification of immunoglobulins and immunoglobulin fragments in the production of antivenoms

15.1 Good manufacturing practices

The purification of immunoglobulins and immunoglobulin fragments for the production of antivenoms should aim at obtaining products of consistent quality, safety and clinical effectiveness. The fractionation processes used should adhere to the GMP principles developed for medicinal products. All operations should therefore be carried out in accordance with an appropriate system of quality assurance and GMP. This covers all stages leading to the finished antivenoms, including the production of water, the production of plasma (animal selection and health control, production of venoms and immunization protocols, containers used for blood and plasma collection, anticoagulant solutions and quality control methods) and the purification, storage, transport, processing, quality control and delivery of the finished product. Of particular relevance is the control of microbiological risks, contamination with particulates and pyrogens, and the existence of a documentation system that ensures the traceability of all production steps. To establish satisfactory traceability of the antivenom produced, all the steps of the purification procedure used for the preparation of the antivenom batch should be recorded carefully in pre-established and approved batch record documents, and sampling should be done at established critical steps for in-process quality control tests.

WHO Guidelines on good manufacturing practices for medicinal products are available (*83*) and the main principles of GMP for the manufacture of blood plasma products of human origin have also been published (*84*, *85*). These Guidelines can serve as a general guide for manufacturing practices in the production of antivenoms. A useful reference in the field of antivenoms is the *Note for guidance on production and quality control of animal immunoglobulins and immunosera for human use* (CPMP/BWP/3354/99) (*86*).

15.2 Purification of the active substance

Antivenoms are prepared from the starting plasma pool using diverse methods to obtain one of the following active substances:

- intact IgG molecules
- $F(ab')_2$ fragments
- Fab fragments.

In general, fractionation procedures should not impair the neutralizing activity of antibodies; they should yield a product of acceptable physicochemical characteristics and purity with a low content of protein aggregates, which is non-pyrogenic and which should provide good recovery of antibody activity. If possible, the process should be simple (with few steps) and economical.

The characteristics of a batch of plasma to be fractionated should be clearly established. The methods used to purify the active substance and the in-process controls should be described in detail in the relevant SOPs. In the following sections, examples of basic protocols used for the production of IgG, $F(ab')_2$ and Fab antivenoms are described. Some additional methodologies introduced to further purify the active substance of antivenoms are also discussed. Variations in these manufacturing procedures have often been developed by individual fractionators and should be considered as acceptable when shown to yield consistently safe and effective preparations of antivenoms.

15.2.1 Purification of intact IgG antivenoms

15.2.1.1 Ammonium sulfate precipitation

In the past, most laboratories that produced whole IgG antivenoms have used fractionation protocols based on salting-out procedures employing ammonium sulfate or sodium sulfate (*87*). Two precipitation steps are included using two different salt concentrations in addition to the elimination of "euglobulins" by precipitation in a diluted acidic solution.

Such fractionation protocols generally lead to a recovery of antibodies of between 40 and 50% and to the formation of protein aggregates. The final product of this procedure used to contain a relatively high proportion of contaminating proteins, such as albumin (*88*). This compromised the safety of the product, since a high incidence of early adverse reactions has been described in response to protein aggregates (*89*).

15.2.1.2 Caprylic acid precipitation

The use of caprylic acid (octanoic acid) as an agent for precipitating proteins from animal plasma has been described in the literature (*90*). Several procedures for the purification of whole IgG antivenoms with a good physicochemical

profile and purity using caprylic acid precipitation of non-immunoglobulin proteins have been developed (*88*, *91*, *92*) and are now used for the production of licensed antivenoms.

Fig. A4.6 illustrates a particular process in which caprylic acid is added slowly to undiluted plasma, with constant stirring, to reach a concentration of 5% (v/v) and pH 5.5. The mixture is stirred at 22–25 °C for a minimum of 1 hour. The precipitated proteins are removed by filtration or centrifugation and discarded. The filtrate or the supernatant containing the immunoglobulins is then submitted to tangential flow filtration to remove residual caprylic acid and low-molecular-mass proteins, depending on the molecular cut-off of the ultrafiltration membranes, and to concentrate the proteins. The immunoglobulin solution is then formulated by adding sodium chloride solution (NaCl), an antimicrobial agent and any other excipient(s) needed, such as stabilizers. The pH is then adjusted to a neutral value and finally subjected to sterile filtration through a filter of pore size 0.22 µm, and dispensed into final containers (vials or ampoules). Variations of this procedure have been introduced by various manufacturers, and include dilution of plasma, changes in caprylic acid concentration, pH, and temperature among others.

Caprylic acid fractionation allows the production of antivenoms of relatively high purity and with a low protein aggregate content, because the immunoglobulins are not precipitated during the process. The yield may reach up to 60–75% of the activity in the starting plasma, depending upon the particular procedure and/or the equipment used. The effectiveness and safety profiles of caprylic acid-fractionated antivenom immunoglobulins have been demonstrated in clinical trials (*89*, *93*, *94*).

15.2.2 Purification of F(ab′)$_2$ antivenoms

Many manufacturers follow the classical protocol for F(ab′)$_2$ antivenom production developed by Pope (*9*, *10*), with a number of recent modifications (*12*, *13*, *95*).

The method of pepsin digestion (see Fig. A4.7) involves the digestion of horse plasma proteins by pepsin, leading to the degradation of many non-IgG proteins, and to the cleavage of IgG into bivalent F(ab′)$_2$ fragments by removal and digestion of the Fc fragment into small peptides. A heating step and the purification of F(ab′)$_2$ fragments by salting out using ammonium sulfate are also key elements of this methodology. Some procedures involve performing the pepsin digestion step on a pre-purified IgG fraction that is obtained by treatment of plasma with ammonium sulfate to obtain an IgG-enriched precipitate, whereas albumin is not precipitated.

Pepsin digestion is accomplished at a pH of 3.0–3.5. A typical protocol is based on incubation at pH 3.3 for 1 hour, at 30–37 °C in a jacketed tank, with a pepsin concentration of 1.0 g/L. Other procedures can be used which give similar

results. Each manufacturer should adjust the pepsin concentration to achieve the required enzymatic activity.

15.2.2.1 Downstream processing using ammonium sulfate

After pepsin digestion, the pH is adjusted to 4.5–5.0 by adding sodium hydroxide (or a weak alkaline buffer; then ammonium sulfate is added with stirring to a final concentration usually close to 12% (w/v). The precipitate is eliminated by filtration or centrifugation, and the filtrate, or supernatant, is heat treated (usually at 56 °C for 1 hour; this is known as "thermocoagulation"). After thermocoagulation, the preparation is cooled down to less than 30 °C, for example, by passing cold water through a jacketed vessel. The resulting fraction is filtered or centrifuged to remove the precipitate. The pH is then adjusted to 7.0–7.2 with sodium hydroxide, and a solution of ammonium sulfate is added with stirring to a final concentration high enough to precipitate the $F(ab')_2$ fragments (usually 23% (w/v) or higher). After an additional filtration step, or following centrifugation, the $F(ab')_2$ precipitate is dissolved, and then desalted (to remove the ammonium sulfate) and concentrated preferentially by tangential flow diafiltration. Care should be taken to avoid aggregate formation by ensuring gentle mixing and rapid dissolving of the precipitate. Alternatively, the 23% (w/v) step is bypassed by some manufacturers and, directly after the heating step, the filtrate obtained is subjected to ultrafiltration. Additional precipitation may also be applied on the starting material at low ionic strength and acid pH to remove "euglobulins" (*10*).

The $F(ab')_2$ solution is then formulated by adding sodium chloride (NaCl), an antimicrobial agent, and any other excipient needed for formulation, such as protein stabilizers, and the pH is adjusted, generally to a neutral value. Finally, the preparation is sterilized by filtration through 0.22 µm filters, and dispensed into final containers (vials or ampoules). Such a process, or similar ones developed by other manufacturers, using pepsin digestion, ammonium sulfate precipitation and tangential diafiltration, is the most often used for the manufacture of $F(ab')_2$ fragments. The yield of this fractionation protocol usually ranges between 30% and 40%.

15.2.2.2 Downstream processing using caprylic acid

Purification of $F(ab')_2$ has also been shown, on an experimental scale, to be achievable by caprylic acid precipitation of non-$F(ab')_2$ proteins after pepsin digestion, with an improved yield (~60%) (*96*). However, the yield obtained on a large scale has not been reported. Fig. A4.8 shows a fractionation scheme for $F(ab')_2$ using caprylic acid. $F(ab')_2$ is not precipitated, therefore reducing the formation of aggregates. Some manufacturers have introduced additional or alternative processing steps such as ion-exchange chromatography or ultrafiltration to eliminate low-molecular-mass contaminants.

15.2.3 Purification of Fab antivenoms

Production of monovalent Fab fragments is performed by some manufacturers (*97*), currently using hyperimmunized sheep plasma. Papain is used to carry out the enzymatic digestion in the presence of L-cysteine as a promoter, and the process of preparation of the fragment may use ammonium sulfate, sodium sulfate or caprylic acid. Fig. A5.9 shows a process in which immunoglobulins are precipitated from plasma by adding ammonium sulfate or sodium sulfate to a concentration of 23%. After filtration the filtrate is discarded and the immunoglobulin precipitate is dissolved in a sodium chloride solution at pH 7.4. Papain is added and digestion performed at 37 °C for 18–20 hours in a jacketed tank. Reaction is stopped by adding iodoacetamide. The product is then applied to a diafiltration system to remove iodoacetamide, salts and low-molecular-mass peptides and equilibrated with a buffered isotonic NaCl solution. The preparation is then chromatographed on an anion exchanger (usually in quaternary aminoethyl (QAE)-based or diethylaminoethyl (DEAE)-based media). Fc fragments and other impurities are bound on the column, whereas Fab fragments pass through. After an additional diafiltration/dialysis step, the product is formulated by adding NaCl, antimicrobial agents (when used) and any other excipients needed, and the pH is adjusted. Finally, the preparation is sterile filtered and dispensed into the final containers.

Fig. A5.6

Example of a fractionation process in which intact IgG is prepared by caprylic acid precipitation of non-immunoglobulin proteins

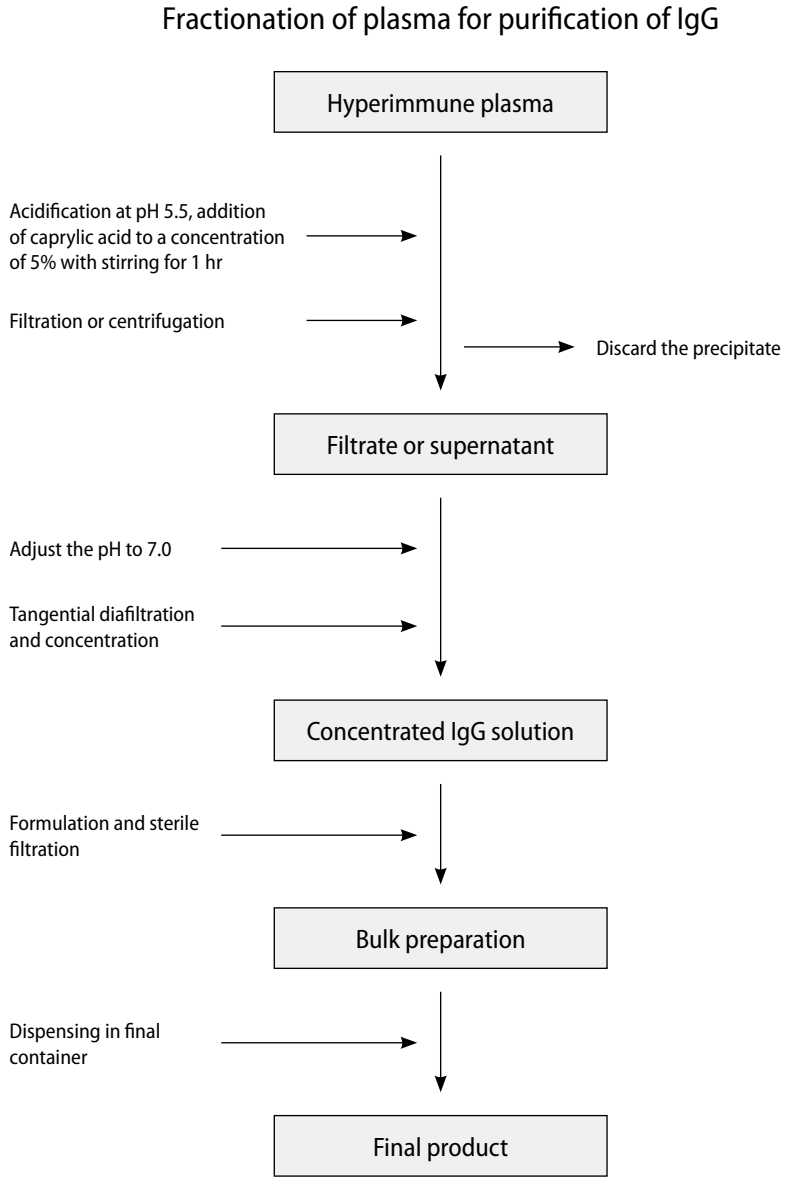


Fig. A5.7

Example of a fractionation process in which $F(ab')_2$ fragments are prepared by pepsin digestion and ammonium sulfate precipitation

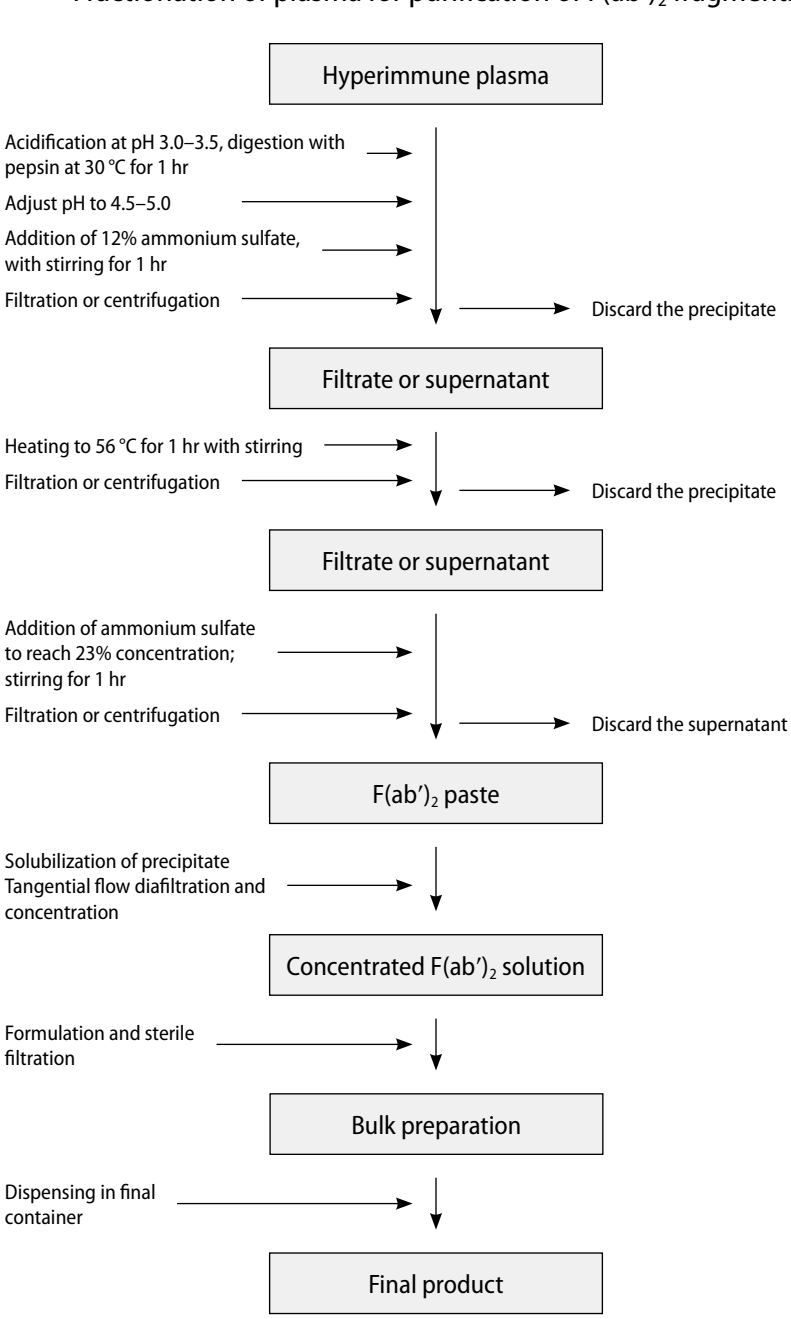


Fig. A5.8

Example of a fractionation process in which F(ab′)$_2$ fragments are prepared by pepsin digestion and caprylic acid precipitation

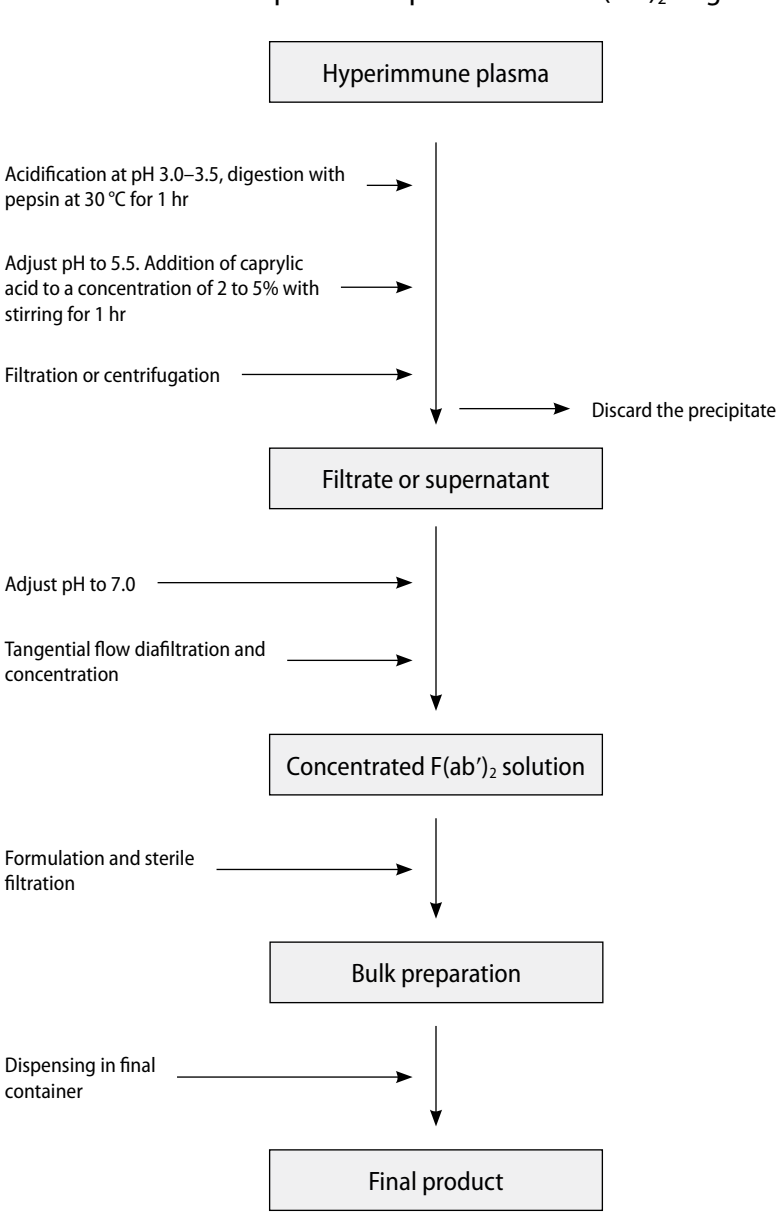
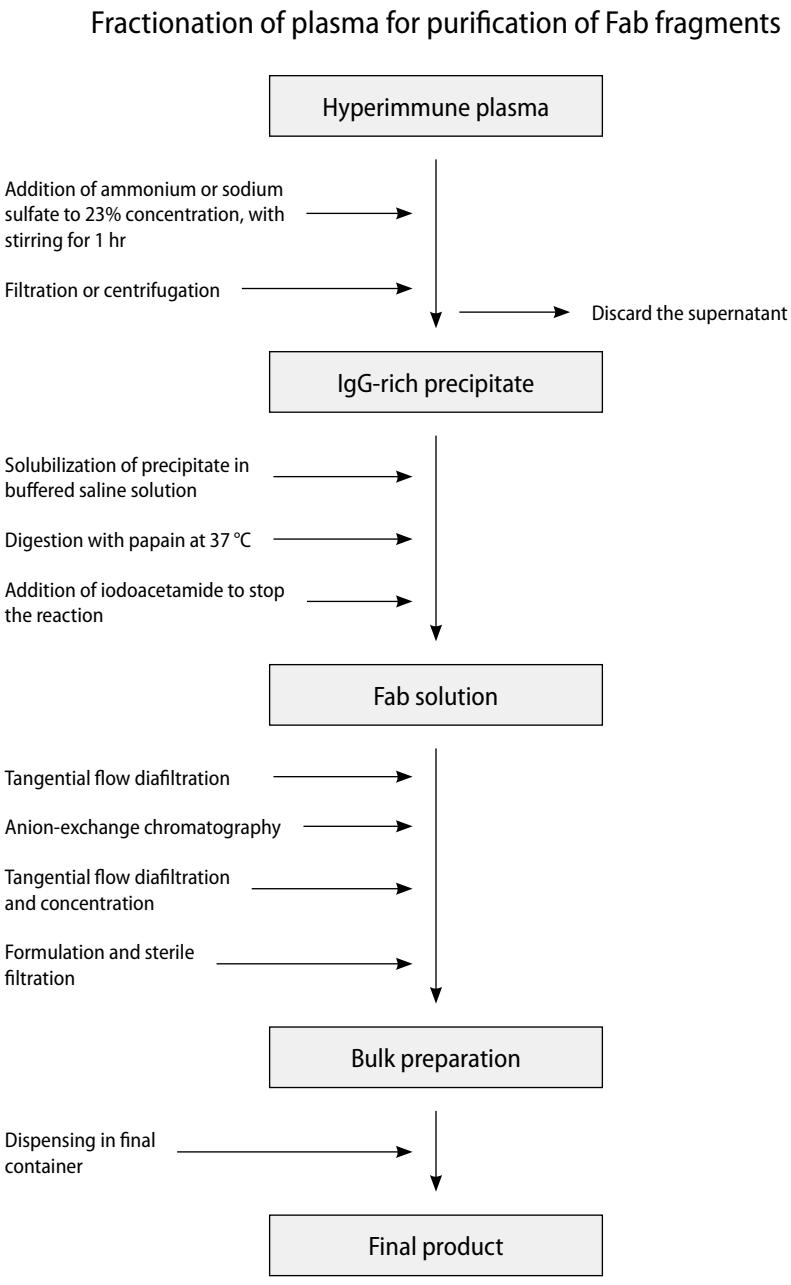


Fig. A5.9

Example of a fractionation process in which Fab fragments are prepared by papain digestion and ammonium sulfate precipitation



15.2.4 Optional additional or alternative steps used by some manufacturers

When performed following GMP and using validated fractionation protocols, the basic methodologies described above for the manufacture of IgG, $F(ab')_2$ and Fab antivenoms allow the production of antivenoms of adequate purity, safety and preclinical efficacy. Nevertheless, some manufacturers include additional steps to enhance product purity. The methodologies include those described below.

15.2.4.1 Ion-exchange chromatography

Ion-exchange chromatography can be successfully used for antivenom purification based on charge differential of the contaminants. Anion-exchange columns of DEAE or QAE gels or membranes, such as quaternary ammonium cellulose microporous membranes, can be used at neutral pH to adsorb protein contaminants and endotoxins (*13*, *95*, *98*). Alternatively, cation-exchange columns, for example, carboxymethyl or sulfopropyl gels, have been used for purification of IgG or $F(ab')_2$ fragments (*96*). The column is equilibrated at acid pH, for example, pH 4.5, to bind the antivenom IgG or its fragments, whereas protein contaminants are eluted in the break-through.

Chromatographic procedures should follow GMP. Columns should be adequately regenerated, sanitized and stored to prolong their useful lifetime. The reproducibility of columns over cycles should be validated. Measures to avoid batch-to-batch contamination should be in place. Specific SOPs should be developed and followed.

15.2.4.2 Affinity chromatography

Affinity chromatography using either immobilized venom or other ligands can be designed to bind immunoglobulins or their fragments (*99*). However, columns usually deteriorate rather rapidly, and meticulous care should be taken to wash, sanitize and store them under appropriate conditions. Procedures should be followed to ensure that any substances leaching from the columns do not affect the quality and safety of the product or else are completely removed during downstream processing; this is especially critical in affinity chromatography using immobilized venom. Affinity processes may affect recovery and high-affinity antibodies may be lost and/or denatured as a result of the harsh elution conditions needed to elute them from the chromatographic material.

15.2.4.3 Process improvement

Some manufacturers have introduced process improvements to enhance the quality or the yield of antivenoms. These include the use of a depth-filtration system combined with filter aids to facilitate filtration steps and improve antivenom recovery. Other additional manufacturing steps may be introduced to ensure inactivation or removal of infectious agents (see section 16).

15.2.5 Formulation

During formulation of antivenoms after diafiltration steps one should consider the addition of salts to adjust the osmolality, addition of preservatives, other excipients, if needed for protein stability, and the adjustment of pH.

In general, antivenoms are formulated at neutral pH (pH 7.0 ± 0.5) although some manufacturers are exploring the feasibility of formulation at more acidic pHs to improve stability and/or to reduce aggregate formation.

Formulation at a pH higher than 7.5 may not be recommended, since the stability of immunoglobulins and their fragments at alkaline pH may be poor, and the formation of aggregates may be favoured.

15.2.6 Analysis of bulk product before dispensing

The biological, physical and chemical characteristics of the final bulk product should meet pre-established specifications before dispensing. Analysis may include tests required to demonstrate:

- the purity and potency of the product
- product sterility
- compliance with the specifications for the aggregate content
- the pyrogen limit and/or the bacterial endotoxin content
- the formulation, that is, the concentration of excipients and the pH.

When the product is a stored liquid, some of these tests (such as the potency assay) may not need to be duplicated on the final container if the processing after the bulk preparation has been validated and shown not to alter this activity.

The sterilization equipment and the integrity of the membrane should be guaranteed before and verified after sterilization; the aseptic filling should also be validated.

15.2.7 Dispensing and labelling of final product

Once compliance of the final bulk product with the quality control specifications is established, the final product is bottled. For this, final glass containers (vials or ampoules) should be used. General principles pertaining to the dispensing of parenteral medicinal products should be applied. The dispensing should be performed in class A (*83*) clean room conditions, usually under a laminar flow hood. The equipment used for dispensing should be calibrated beforehand to ensure that the correct volume is delivered. European GMPs now recommend that sterile filtration be carried out at the closest point immediately before filling.

In the case of ampoules, the dispensing system should ensure an aseptic closure and the sealing of the ampoule should prevent risk of protein denaturation

due to heat. For vials, insertion of rubber stoppers should be done inside this clean dispensing area. The quality of the rubber stoppers should be such as to guarantee inertness and to prevent leaching. Thereafter, aluminium seals should be placed on each vial in a clean area outside the class A area. Ampoules or vials containing the final product should then be properly identified and stored in a quarantine area maintained under proper storage conditions. Samples of the antivenoms should be sent to the quality control laboratory for analysis.

When an antivenom complies with all the quality control tests established for the final product, it should be properly labelled and identified.

- The vial or ampoule should be labelled with, at least, the following information:
  - (a) name of the product and of the producer;
  - (b) animal species used to produce the antivenom;
  - (c) batch number;
  - (d) pharmaceutical presentation (liquid or freeze-dried);
  - (e) volume content;
  - (f) administration route;
  - (g) specificity – venoms neutralized by the antivenom, including both the common and the scientific name of the snake(s);[9]
  - (h) neutralizing potency;
  - (i) storage conditions; and
  - (j) expiry date.

Additional information may be requested by NRAs.

- The package, which is usually a cardboard box, in which the vials or ampoules are packed, should include the same information as is given on the primary container.
- The package insert should include all the information relating to the product, as established by NRAs, including:
  - (a) the neutralizing potency;
  - (b) the recommended dosage;
  - (c) reconstitution procedure, if lyophilized;
  - (d) the mode of administration (for example, the dilution of antivenom in a carrier fluid such as saline);

[9] Special care should be taken to consider potential changes in snake species taxonomy.

(e) the rate of administration;
(f) details on the symptoms and treatment of early and delayed adverse reactions;
(g) snake species against which the antivenom is effective;
(h) recommended storage conditions; and
(i) an indication that the product is for single use.

15.2.8 Use of preservatives

The addition of preservatives to prevent bacterial and fungal contamination should be kept to a minimum during plasma storage and during fractionation. Their inclusion during the manufacturing process should be clearly justified, and should never substitute for any aspect of GMP. Preservatives can be considered in the final product, especially if it is manufactured in liquid form. Antimicrobial agents currently used in antivenom formulation include phenol and cresols. In general, phenol concentration is adjusted to 2.5 g/L, and concentration of cresols should be less than 3.5 g/L. The concentration of preservatives should be validated by each production laboratory on the basis of assays to test their efficacy and keeping in mind that they may degrade with time and cease to be effective. It is necessary to ascertain that any agent used has no potential detrimental interaction with the active substance and excipients of antivenoms. Any change in the formulation involving preservatives, or the elimination of preservatives from the final product, requires a very careful risk–benefit assessment on various microbial safety aspects, as well as a detailed validation procedure. Mercury-containing preservatives are not recommended in antivenom manufacture. The volume of antivenom required for the treatment of envenoming (in excess of 50 mL) might lead to an exposure to mercury far higher than the amounts currently used for other biological preparations and the levels at which they are toxic, especially in young children, are not known (*100*, *101*).

15.2.9 Freeze-drying

Antivenoms are available either as liquid or as freeze-dried preparations. Freeze-dried antivenoms, which may usually be stored at a temperature not exceeding 25 °C, are generally distributed to markets where the cold chain cannot be guaranteed, such as in many tropical regions of the world. The absence of guarantee of a cold chain during distribution highlights the need for manufacturers to demonstrate the stability of the antivenoms under the high temperatures found in tropical climates.

Freeze-drying is a critical operation. Careful attention should be paid to the rate of freezing as well as to the protocol used for the primary and secondary drying cycles (*102*). The details of the freeze-drying protocols are product-

specific and should be adjusted according to the particular formulation of each antivenom. Inadequate freeze-drying protocols may affect the physicochemical quality of the product, inducing protein precipitation and denaturation, as well as aggregate formation, and altering stability and reconstitution. Specific stabilizers, such as sugars or polyols, aimed at protecting proteins from denaturation and aggregation, may be added to the final formulation of the antivenom (*103*). Bulking agents, frequently used for some biological products, are generally not required in antivenoms owing to their relatively high protein concentration; however, they are sometimes used for high-titre monospecific antivenoms.

15.2.10 Inspection of final container

All of the vials or ampoules in each batch of liquid antivenoms should be inspected, either visually or using a mechanical device. Any vial or ampoule presenting turbidity, abnormal coloration, presence of particulate matter, or defects of the vial, stopper or capsule should be discarded. In the case of freeze-dried products, a representative sample of the whole batch should be first tested dry for meltback and contamination with foreign matter, then dissolved in the solvent and inspected further as described. Turbidity can be assessed quantitatively using a turbidimeter.

15.2.11 Archive samples of antivenoms

In compliance with GMP, manufacturing laboratories should archive a number of vials of each antivenom batch, under the recommended storage conditions, in an amount that would enable the repetition of all quality control tests, when required.

15.3 Pharmacokinetic and pharmacodynamic properties of IgG, $F(ab')_2$ and Fab

Owing to their different molecular mass, the pharmacokinetics of heterologous IgG molecules (approximately 150 kDa) and $F(ab')_2$ (approximately 100 kDa) and Fab (approximately 50 kDa) fragments differ significantly. In envenomed patients, Fab fragments have the largest volume of distribution and readily reach extravascular compartments. Fab fragments are, however, rapidly eliminated, mainly by renal excretion, thus having a short elimination half-life (from 4–24 hours) (*104*, *105*). In contrast, $F(ab')_2$ fragments and intact IgG molecules are not eliminated by the renal route (they are eliminated by phagocytosis and opsonized by the reticuloendothelial system) and therefore have a longer elimination half-life (between 2 and 4 days) (*20*, *106*, *107*). Such different pharmacokinetic profiles have important pharmacodynamic implications, and the selection of the ideal type of active substance in an antivenom should rely on a careful analysis of the venom toxicokinetics and antivenom pharmacokinetics.

Another difference between low-molecular-mass fragments, such as Fab and those with a higher molecular mass, such as $F(ab')_2$ and IgG, is the number of paratopes of each molecule: Fab has one antigen-binding site whereas IgG and $F(ab')_2$ each have two binding sites. Thus they will be able to form large and stable complexes or precipitates with antigens carrying several epitopes, while Fab will form small, reversible non-precipitable complexes.

Ideally, the volume of distribution of an antivenom should be as similar as possible to the volume of distribution of the main toxins in a particular venom; however, this is rarely the case. In venoms composed of low-molecular-mass toxins, such as some elapid snake venoms, low-molecular-mass neurotoxins are rapidly absorbed into the bloodstream and are rapidly distributed to the extravascular spaces where toxin targets are located. Furthermore, low-molecular-mass toxins are eliminated from the body in a few hours. In these cases, an antivenom of high distribution volume that readily reaches extravascular spaces, such as Fab, might be convenient, although its action is then eliminated within a few hours. It should be noted, however, that a number of elapid venoms contain some high-molecular-mass toxins of great clinical significance, such as procoagulants and pre-synaptic phospholipase A2 neurotoxins.

In contrast, in the case of viperid snake venoms and other venoms made up of toxins of larger molecular mass, including a number of elapid venoms, many of which act intravascularly to provoke bleeding and coagulopathy, the situation is different. The time required for toxins to distribute to extravascular spaces is longer than in the case of low-molecular-mass neurotoxins, and the targets of some of these toxins are present in the vascular compartment. In addition, the toxins of viperid venoms have a long half-life in vivo and can remain in the body for several days (*108*, *109*). In this case, an antivenom made by Fab fragments neutralizes the toxins that reach the circulation but, after a certain time has elapsed, the Fab fragments are eliminated and non-neutralized toxins reach the circulation. This gives rise to the well-known phenomenon of recurrent envenoming, that is, the reappearance of signs and symptoms of envenoming at later time intervals after the initial control of envenoming. This situation demands repeated administration of antivenom to maintain therapeutic levels of Fab in the circulation (*110*). Therefore, in such envenomings, antivenoms made of IgG or $F(ab')_2$ may be more appropriate because of their longer elimination half-lives. Moreover, it has been proposed that formation of venom–antivenom complexes in the circulation results in the redistribution of venom components from the extravascular space to the blood compartment, where they are bound and neutralized by circulating antivenom, provided that the dose of antivenom is sufficient (*111*, *112*). Consequently, the maintenance of a high concentration of specific antivenom antibodies in the circulation for many hours is required for complete neutralization of toxins

reaching the bloodstream during both early and late phases of envenoming (redistribution of toxins) present in the extravascular space. In conclusion, IgG and $F(ab')_2$ antivenoms have a pharmacokinetic profile that makes them more effective in many types of snake-bite envenoming.

15.4 Main recommendations

- **Antivenoms should be manufactured using fractionation procedures that are well established, validated, and shown to yield products with proven safety and effectiveness. Fractionation processes used for the manufacture of antivenoms should adhere to the principles of GMP for parenteral medicinal products.**
- **Antivenoms can be composed of intact IgG molecules, $F(ab')_2$ fragments or Fab fragments. Intact IgG antivenoms are mainly produced by caprylic acid precipitation of non-IgG plasma proteins, leaving a highly purified IgG preparation in the supernatant or filtrate.**
- **$F(ab')_2$ fragment antivenoms are produced by pepsin digestion of plasma proteins, at acidic pH, usually followed by $F(ab')_2$ purification by salting out with ammonium sulfate solutions or by caprylic acid precipitation. Fab monovalent fragments are obtained by papain digestion of IgG at neutral pH.**
- **Further to ultrafiltration to remove low-molecular-mass contaminants, preparations are formulated, sterilized by filtration and dispensed in the final containers. Formulations of antivenoms may include preservative agents. Additional steps, such as chromatography, can be added to the fractionation protocols to enhance purity.**
- **Antivenoms can be presented as liquid or freeze-dried preparations. Freeze-drying of antivenoms should be performed in conditions that ensure no denaturation of proteins and no formation of protein aggregates.**
- **IgG, $F(ab')_2$ and Fab antivenoms exhibit different pharmacokinetic profiles: Fab fragments have a larger distribution volume and a much shorter elimination half-life. Thus, for viperid envenomings, IgG or $F(ab')_2$ antivenoms have a more suitable pharmacokinetic profile, whereas Fab fragments may be useful for the neutralization of venoms rich in low-molecular-mass neurotoxins which are rapidly distributed to the tissues. However, in general terms, IgG and $F(ab')_2$ antivenoms have shown a better pharmacokinetic profile than Fab antivenoms.**

16. Control of infectious risks

16.1 Background

The viral safety of any biological product results from a combination of measures to ensure a minimal risk of viral contamination in the starting raw material (plasma), together with steps to inactivate or remove potential contaminating viruses during processing.

There are currently several recognized complementary approaches used for virus risk reduction for biological products. These are:

- minimizing the potential initial virus content by implementing a quality system for the production of the raw material;
- contribution of the manufacturing processes to inactivating and/or removing residual viruses during manufacture of the biological product; such a contribution can be inherent to the existing production technology or may result from the introduction of dedicated viral reduction steps;
- adherence to GMP at all steps of the manufacturing process;
- appropriate and timely response to any infectious events recognized during the clinical use of the product.

Production steps to inactivate and/or remove viruses have long been shown to play a powerful role in ensuring the safety of biologicals (*84*). Similarly, keeping to a minimum the potential viral load at the stage of the plasma pool, through appropriate epidemiological surveillance and health control of the donor animals, is also an important safety measure (see section 12).

Based on experience with human plasma products, a production process for antivenoms that includes two robust steps for viral reduction (preferably comprising at least one viral inactivation step) should provide a satisfactory level of viral safety. However, it should be kept in mind that non-enveloped viruses are more difficult to inactivate or remove than lipid-enveloped viruses.

16.2 Risk of viral contamination of the starting plasma

The main structural characteristics of viruses reported to possibly infect horses, sheep and goats are presented in Tables A5.7 and A5.8. They include viruses with a DNA or RNA genome, with and without a lipid envelope, and vary widely in size (22 to 300 nm).

A few of these viruses have been identified as possibly present, at least at some stages of the infection cycle, in the blood, or are considered pathogenic to humans. Special attention should be paid to these viruses.

16.3 Viral validation of manufacturing processes

An understanding of how much a manufacturing process may contribute to the viral safety of antivenoms is fundamental to both manufacturers and regulators. Such an understanding can only be achieved by viral validation studies. These studies are complex and require well-established virology laboratory infrastructure and cell culture methodologies. They are usually carried out by specialized laboratories, outside the manufacturing facilities. The principles guiding such studies have been described in WHO Guidelines (*84*) and are summarized below.

Table A5.7
Viruses identified in horses (*86*, *113*)

Virus	Family	Size (nm)	Genome[a]	Presence in blood reported[b]	Classified as pathogenic to humans *(86)*
Lipid-enveloped viruses					
Borna virus[c]	Bornaviridae	70–130	ss-RNA	Yes	
Equine arteritis virus	Arteriviridae	50–60	ss-RNA		
Equine encephalitis virus, Eastern and Western	Togaviridae	40–70	ss-RNA		Yes
Equine coronavirus	Coronaviridae	75–160	ss-RNA		
Equine foamy virus	Retroviridae	80–100	ss-RNA	Yes	
Equine herpesvirus 1–5	Herpesviridae	125–150	ds-DNA		Yes
Equine infectious anaemia virus	Lentiviridae	80–100	ss-RNA	Yes	
Equine influenza virus	Orthomyxoviridae	80–120	ss-RNA		Yes

Table A5.7 *continued*

Virus	Family	Size (nm)	Genome[a]	Presence in blood reported[b]	Classified as pathogenic to humans *(86)*
Equine morbillivirus (Hendra virus)	Paramyxoviridae	150	ss-RNA		Yes
Japanese encephalitis virus	Flaviviridae	40–70	ss-RNA		Yes
Equine hepacivirus	Flaviviridae	40–70	ss-RNA	Yes (*114*)	
Equine pegivirus	Flaviviridae	40–70	ss-RNA	Yes (*115*)	
Nipah virus	Paramyxoviridae	150–300	ss-RNA		Yes
Rabies virus	Rhabdoviridae	75–180	ss-RNA		Yes
Salem virus	Paramyxoviridae	150–300	ss-RNA		
St Louis encephalitis virus	Flaviviridae	40–70	ss-RNA		Yes
Theiler's disease-associated virus	Flaviviridae	40–70	ss-RNA	No (*114*)	
Tick-borne encephalitis virus (*116*, *117*)	Flaviviridae	40–70	ss-RNA		Yes
Venezuelan equine encephalitis virus	Togaviridae	40–70	ss-RNA	Yes	Yes
Vesicular stomatitis virus	Rhabdoviridae	50–80	ss-RNA	Yes	Yes
West Nile virus	Flaviviridae	40–70	ss-RNA	Yes	Yes

Table A5.7 *continued*

Virus	Family	Size (nm)	Genome[a]	Presence in blood reported[b]	Classified as pathogenic to humans *(86)*
Non-lipid-enveloped viruses					
Equine encephalosis viruses	Reoviridae	80	ds-RNA		
Equine rhinitis A and B viruses	Picornaviridae	22–30	ss-RNA		
Equine rotavirus	Reoviridae	60–80	ds-RNA		

[a] ss-RNA, single-stranded ribonucleic acid; ds-DNA, double-stranded deoxyribonucleic acid; ds-RNA, double-stranded ribonucleic acid.

[b] Absence of a report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection.

[c] Recent studies have suggested that Borna virus is non-pathogenic to humans (*118*).

Table A5.8
Viruses identified in sheep and goats (*86*)

Virus	Family	Size (nm)	Genome[a]	Reported presence in blood[b]	Classified as pathogenic to humans (*86*)
Lipid-enveloped viruses					
Adenovirus	Adenoviridae	80–110	ds-DNA		
Akabane virus	Bunyaviridae	80–120	ss-RNA		
Bluetongue virus	Reoviridae	80	ds-RNA		Yes
Border disease virus	Flaviviridae	40–70	ss-RNA		
Borna virus[c]	Bornaviridae	70–130	ss-RNA	Yes	
Bovine herpesvirus types 1, 2, 4	Herpesviridae	120–200	ds-DNA		

Table A5.8 *continued*

Virus	Family	Size (nm)	Genome[a]	Reported presence in blood[b]	Classified as pathogenic to humans (*86*)
Bovine viral diarrhoea virus	Togaviridae	40–60	ss-RNA		
Loiping ill virus	Flaviviridae	40–50	ss-RNA		Yes
Nairobi sheep disease	Bunyaviridae	80–120	ss-RNA		
Ovine/bovine papillomavirus	Papillomaviridae	40–55	ds-DNA		
Ovine herpesvirus 2	Herpesviridae	120–200	ds-DNA		
Parainfluenza virus type 3	Paramyxoviridae	150–300	ss-RNA		Yes
Peste des petits ruminants (Morbillivirus)	Paramyxoviridae	150–300	ss-RNA		
Poxviruses (Parapox, Capripox, Cowpox)	Poxviridae	140–260	ds-DNA		Yes
Respiratory syncytial virus	Paramyxoviridae	150–300	ss-RNA		
Retroviruses (Caprine arthritis encephalitis virus, Maedi-Visna virus, Jaagsiekte virus, Bovine leukaemia virus	Retroviridae	80–100	ss-RNA		

Table A5.8 *continued*

Virus	Family	Size (nm)	Genome[a]	Reported presence in blood[b]	Classified as pathogenic to humans (*86*)
Rift Valley fever complex	Bunyaviridae	80–120	ss-RNA		Yes
Ross river virus	Togaviridae	70	ss-RNA		
Rotavirus	Reoviridae	80	ds-RNA		
Tick-borne encephalitis virus	Flaviviridae	40–50	ss-RNA		Yes
Vesicular stomatitis virus	Rhabdoviridae	50–380	ss-RNA	Yes	Yes
Wesselbron virus	Flaviviridae	40–50	ss-RNA		Yes
Non-lipid-enveloped viruses					
Epizootic haemorrhagic disease virus	Reoviridae	80	ds-RNA		
Foot and mouth disease virus	Picornaviridae	27–30	ss-RNA		Yes
Reovirus 1-3	Reoviridae	60–80	ds-RNA		

[a] ds-DNA, double-stranded deoxyribonucleic acid; ss-RNA, single-stranded ribonucleic acid; ds-RNA, double-stranded ribonucleic acid.

[b] Absence of a report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection.

[c] Recent studies have suggested that Borna virus is non-pathogenic to humans (*118*).

16.3.1 Down-scale experiments

The contribution of manufacturing processes to inactivation and/or removal of potential viral contamination should be demonstrated. For this purpose, viral validation studies should be performed using at least three viruses exhibiting different structural characteristics. The antivenom manufacturer should first identify the steps that, based on the existing literature, are likely to remove or inactivate viruses and, then, provide evidence and quantitative assessment of the extent of virus reduction achieved for the specific process evaluated.

Validation should be done by down-scale experiments. The accuracy of the down-scale process should be assessed by comparing the characteristics of the starting intermediate and the fraction resulting from that step, for both the laboratory and the production scales. It may be more appropriate to use manufacturing intermediates for spiking in viral validation studies. Selected physical factors (for example, temperature, stirring or filtration conditions) and chemical factors (for example, pH or concentration of precipitating agents such as caprylic acid) should be as close as possible to those used at manufacturing scale.

Once the step is accurately modelled, the antivenom fraction derived from the fractionation process just prior to the step being evaluated (for example, the starting plasma to be subjected to a low pH treatment, or to caprylic acid precipitation, or a $F(ab')_2$ fragment fraction to be subjected to ammonium sulfate heat treatment) should be spiked with one of the model viruses selected. Viral infectivity, most often determined using cell culture assays (less frequently animal models), should be quantified before (for example, prior to pH adjustment and addition of pepsin) and immediately after (for example, following low pH adjustment and incubation at that pH for a known period of time in the presence of pepsin) the steps evaluated to determine the viral clearance achieved. The results are conventionally expressed as the logarithm (log) of the reduction in infectivity that is observed. Total infectivity or viral load is calculated as the infectious titre (infectious units per mL) multiplied by the volume. For a viral inactivation step, it is highly recommended that the kinetics of the virus kill be evaluated. Such inactivation kinetics of the infectivity provide an important indication of the virucidal potential of the step and enables comparison of the data obtained to those from published studies.

Typically, a viral reduction of 4 logs or more is considered to represent an effective and reliable viral safety step.

Establishing the relative insensitivity of a manufacturing step to changes or deviations in process conditions is also important in evaluating its robustness, in addition to adding to the level of understanding of its contribution to the overall viral safety of the preparation. This can be achieved by validating the same step using a range of conditions deviating from those used in production (such as an upper pH limit applied to a pepsin digestion or to a caprylic acid precipitation step).

Virus validation studies are subject to a number of limitations (*84*), which should be considered when interpreting the results.

16.3.2 Selection of viruses for the validation of antivenom production processes

Viruses selected for viral validation studies should resemble as closely as possible those which may be present in the starting animal plasma material (Tables A5.7 and A5.8). It is usual to select a wide variety of viruses, some enveloped and some non-enveloped. At least 1–2 non-enveloped, relatively small viruses should be selected for validation. When possible, viruses known to potentially contaminate animal plasma (called "relevant viruses") should be used.

Table A5.9 gives examples of a few viruses that have been used for the validation of animal-derived immunoglobulins. Vesicular stomatitis virus (VSV) and West Nile virus (WNV) are relevant lipid-enveloped horse plasma-borne viruses. Bovine viral diarrhoea virus (BVDV), a lipid-enveloped flavivirus, can be used as a model for WNV, tick-borne encephalitis virus, and for the Eastern, Western, and Venezuelan equine encephalitis togaviruses. Pseudorabies virus is a lipid-enveloped virus that can serve as a model for pathogenic equine herpesvirus. Encephalomyocarditis virus (EMCV), a picornavirus, can serve as a model for non-lipid-enveloped viruses. Porcine parvovirus can also be selected as a model for small resistant non-lipid-enveloped viruses or as a relevant virus when pepsin of porcine origin is used in the manufacture of $F(ab')_2$ fragments.

This list is not exhaustive and other model viruses can be used for validation studies of animal-derived antivenoms, in particular taking into account the characteristics of the viruses that may be present in the animal species used to generate antivenoms.

Table A5.9
Examples of laboratory model viruses that can be used for validation studies of horse-derived antivenoms

Virus	Family	Lipid-enveloped	Size (nm)	Genome[a]	Resistance	Model for
Animal parvovirus (for example, porcine)	Parvoviridae	No	18–26	ss-DNA	High	Relevant virus (when pepsin of porcine origin is used)
Bovine viral diarrhoea virus	Togaviridae	Yes	40–60	ss-RNA	Low	Eastern, Western and Venezuelan equine encephalitis virus; tick-borne encephalitis virus
Parainfluenza virus	Paramyxoviridae	Yes	100–200	ss-RNA	Low	Hendra virus; Nipah virus; Salem virus
Poliovirus; encephalomyocarditis virus; hepatitis A virus	Picornaviridae	No	25–30	ss-RNA	Medium-high	Equine rotavirus
Pseudorabies virus	Herpes	Yes	100–200	ds-DNA	Medium	Equine herpesvirus
Reovirus type 3	Reoviridae	No	60–80	ds-RNA	Medium	Equine encephalosis virus
Sindbis virus	Togaviridae	Yes	60–70	ss-RNA	Low	Eastern, Western and Venezuelan equine encephalitis virus
Vesicular stomatitis virus	Rhabdoviridae	Yes	50–200	ss-RNA	Low	Relevant virus
West Nile virus	Flaviridae	Yes	40–70	ss-RNA	Low	Relevant virus and model for Eastern equine encephalitis virus

[a] ss-DNA, single-stranded deoxyribonucleic acid; ss-RNA, single-stranded ribonucleic acid; ds-DNA, double-stranded deoxyribonucleic acid; ds-RNA, double-stranded ribonucleic acid.

16.4 Viral validation studies of antivenom immunoglobulins

There is no documented case of transmission of zoonotic infections, including viral diseases, by antivenom immunoglobulins, or any other animal-derived immunoglobulins. Absence of reports of viral transmission may result from a lack of long-term surveillance of the patients receiving antivenoms. Alternatively, this may reveal that current processes for the manufacturing of antivenoms include processing steps that contribute to viral safety.

Among the various processing steps used in the production of antivenoms, caprylic acid and low pH treatments are known to contribute to safety against lipid-enveloped viruses. This information is based on well-established experience in the fractionation of human plasma with a production step comprising caprylic acid (*119–121*) or low pH treatment (*84*, *122–124*).

Although information is still limited, there is growing evidence that similar steps used in the production of animal-derived immunoglobulins may also inactivate or remove viruses. In addition, some manufacturers have implemented dedicated viral reduction procedures. After the introduction of a new step in the process, the stability of the product must be verified.

16.4.1 Caprylic acid treatment

The conditions used for caprylic acid treatment of antivenoms (*88*, *113*) and of human immunoglobulins (*119–121*) are similar, in particular the pH range, duration of treatment, temperature, and the caprylic acid/protein ratio, as summarized in Table A5.10.

Table A5.10
Comparison of conditions for caprylic acid treatment used for human immunoglobulin preparations and antivenoms (*113*)

Product	Protein concentration (g/L)	Caprylic acid (g/kg solution)	pH	Temperature (°C)	Duration (hours)
Human IgG	35	7.45	5.5	22	1
Human IgM enriched	43	15	4.8	20	1
Human IgM	25	20	5.0	20	1
Antivenoms	60–90	50	5.5–5.8	18–22	1

16.4.1.1 Validation studies with human immunoglobulins

Unsaturated fatty acids, most specifically caprylic acid, have long been known to have the capacity to inactivate lipid-enveloped viruses in human plasma protein fractions (*125*, *126*). The non-ionized form of caprylic acid is thought to disrupt the lipid bilayer and membrane-associated proteins of enveloped viruses. Utilizing the dissociation reaction and varying the concentration of the ionized form of caprylate, a specific amount of the non-ionized form of caprylate can be maintained over a wide pH range.

The robustness of caprylic acid treatment applied to human IgG, human IgM and IgM-enriched preparations has been investigated using various enveloped viruses (human immunodeficiency virus (HIV), BVDV, Sindbis virus and Pseudorabies virus (*120*). Under the conditions applied during manufacture, caprylic acid leads to robust inactivation of lipid-enveloped viruses; pH is a particularly critical parameter and should be less than 6.

Another investigation studied the viral reduction achieved during treatment by caprylate of a human IgG product (119). At pH 5.1, 23 °C, in the presence of 9 mM caprylate, ≥ 4.7 and ≥ 4.2 log of HIV and pseudorabies virus, respectively, were inactivated during the 1 hour treatment, but only 1.5 log for BVDV was inactivated. At 12 mM caprylate, ≥ 4.4 log of BVDV were inactivated within this period. At pH 5.1, 24 °C, and 19 mM caprylate, and pH 5.1, 24 °C, and 12 mM caprylate, complete inactivation of BVDV and of HIV and pseudorabies virus was achieved in less than 3 minutes.

Treatment of cryoprecipitate-poor plasma with 5% caprylic acid/pH 5.5 at 31 ± 0.5 °C (a condition close to that used to prepare antivenoms) was shown to inactivate ≥ 5 log of HIV, BVDV and pseudorabies virus in less than 5 min (*127*).

16.4.1.2 Validation studies with antivenom immunoglobulins

Virus inactivation studies have been carried out on an $F(ab')_2$ fraction obtained from pepsin-digested plasma subjected to ammonium sulfate precipitation. The $F(ab')_2$ fraction was subjected to precipitation by drop-wise addition of caprylic acid to 0.5% (final concentration) and the mixture was maintained under vigorous stirring for 1 hour at 18 °C. Rapid and complete reduction of BVDV, pseudorabies virus and VSV (> 6.6 $\log_{10}$, > 6.6 $\log_{10}$, and > 7.0 $\log_{10}$, respectively) was observed. No significant reduction (0.7 $\log_{10}$) of the non-enveloped EMCV (*126*) was observed.

In another process used to prepare equine immunoglobulins, serum is thawed at 4 °C, subjected to heating at 56 °C for 90 minutes, brought to 20 ± 5 °C, adjusted to pH 5.5 and subjected to 5% caprylic acid treatment for 1 hour. This process led to fast reduction of infectivity of > 4.32 and

> 4.65 $\log_{10}$ for pseudorabies virus and BVDV, respectively. The caprylic acid step was confirmed to have only limited impact on the infectivity of EMCV and minute virus of mice (MVM) non-lipid-enveloped viruses (*128*). Data suggest that significant reduction in the infectivity of lipid-enveloped viruses can be obtained during caprylic acid treatment of antivenoms. The reduction of viral infectivity may result from both viral inactivation and partitioning during the precipitation step. No significant inactivation of non-enveloped viruses is expected.

16.4.1.3 Recommended actions

Further studies of the viral reduction achieved during caprylic acid treatment of antivenoms are recommended; in particular, robustness studies to define the impact on process variations should also be performed.

16.4.2 Acid pH treatment

The conditions used for low pH treatment of equine-derived antivenom immunoglobulins and of human immunoglobulins are summarized in Table A5.11.

Table A5.11
Typical conditions for acid pH treatment of human IgG preparations and equine antivenoms (*113*)

Product	Protein concentration (g/L)	pH	Temperature (°C)	Duration (hours)
Human IgG	40–60	4.0	30–37	20–30
Antivenoms	60–90	3.1–3.3	30–37	0.6–24

16.4.2.1 Validation studies with human immunoglobulins

Many studies have demonstrated that the low pH 4 treatment used in the manufacture of human intravenous IgG has the capacity to inactivate lipid-enveloped viruses (122–124). The rate and extent of inactivation may differ depending upon the virus. Inactivation is temperature dependent, and is influenced by the formulation of the IgG solution. Pepsin is sometimes added in trace amounts (to reduce anticomplementary activity and content of aggregates) but, at this low concentration, contributes little to virus kill (86). Most non-lipid-enveloped viruses are resistant to acid pH treatment.

16.4.2.2 Virus inactivation studies performed with antivenom immunoglobulins

As described in section 15, peptic cleavage of horse plasma IgG at pH 3.0–3.3 for 60 minutes is a common procedure for the preparation of $F(ab')_2$. More than 4 logs of inactivation of WNV and of Sindbis virus has been observed in horse plasma subjected to peptic digestion at pH 3.2 for 60 minutes (*129*). WNV was very sensitive whether pepsin was added or not, whereas the rate and extent of inactivation of Sindbis virus was higher in the presence of pepsin. This suggests that pH 3.2 alone inactivates WNV, while other phenomena involving the action of pepsin contribute to Sindbis virus inactivation at low pH.

Confirmation of the significant inactivation of lipid-enveloped viruses during peptic cleavage of plasma at pH 3.2 was obtained by another study (*126*). In this study process, plasma was diluted with two volumes of saline, pH was adjusted to 3.3, and pepsin was added to a final concentration of 1 g/L. The mixture was incubated at pH 3.3 for 1 hour. Inactivation of pseudorabies virus > 5.1 $\log_{10}$ occurred in less than 6 minutes and > 7.0 $\log_{10}$ in 60 minutes. There was > 3.1 $\log_{10}$ and > 4.5 $\log_{10}$ inactivation of VSV after 6 and 20 minutes, respectively. The reduction of infectivity of BVDV was less: 1.7 $\log_{10}$ after 60 minutes. Inactivation of EMC, a non-enveloped virus, was relatively slow but reached between 2.5 and 5.7 $\log_{10}$ after 60 minutes of pepsin incubation. This showed that reduction of infectivity of at least some non-lipid-enveloped viruses may take place during peptic digestion of diluted horse plasma. This does not mean, however, that other non-lipid-enveloped viruses would be inactivated to the same extent under such conditions.

16.4.2.3 Recommended actions

Manufacturers of $F(ab')_2$ antivenoms must validate the pepsin digestion process since virus inactivation is likely to be influenced by pH, time, temperature, pepsin content and protein content. Robustness studies to define the impact on process variations are also recommended.

16.4.3 Filtration steps

Other steps used in antivenom production may contribute to viral safety through nonspecific virus removal. The virus removal capacity of two depth-filtration steps performed in the presence of filter aids and used in the production of equine-derived immunoglobulins prepared by ammonium sulfate precipitation of pepsin-digested IgG has been evaluated (*130*). Clearance factors of 5.7 and 4.0 $\log_{10}$ have been found for two lipid-enveloped viruses (infectious bovine rhinotracheitis virus and canine distemper virus, respectively) and of 5.3 and 4.2 $\log_{10}$ for two non-lipid-enveloped viruses (canine adenovirus virus and poliovirus type I, respectively). However, it should be kept in mind that viral reductions obtained by non-dedicated removal steps are usually regarded

as less robust than those resulting from dedicated viral inactivation or removal steps (*84*).

16.4.4 Validation of dedicated viral reduction treatments

16.4.4.1 Pasteurization

Pasteurization is defined as the treatment of a liquid protein fraction for 10 hours, usually at 60 °C. It is a well-established viral inactivation treatment of human plasma products, such as IgG (*84*). It is being used in the production process of a few equine-derived immunoglobulins (*13*).

Validation studies have shown that heating a purified equine immunoglobulin at 58 °C ± 0.1 °C without stabilizers inactivates ≥ 4.8 $\log_{10}$ of pseudorabies virus and ≥ 4.3 $\log_{10}$ of BVDV in less than 30 minutes, and > 4.7 log of EMCV in less than 1 hour. In contrast, infectivity of MVM, a non-enveloped virus, was still detected after 9 hours and 30 minutes of treatment; only 1.59 $\log_{10}$ were inactivated (*128*).

16.4.4.2 Nanofiltration

Nanofiltration is a technique of filtration specifically designed to remove viruses, based on size, while permitting flow-through of the desired protein (*131*). Effective virus removal requires, in principle, that the pore size of the filter be smaller than the effective diameter of the virus particles.

16.4.5 Other viral inactivation treatments currently not used in antivenom manufacture

Other methods of viral inactivation have been developed to ensure the viral safety of biological products. These include, in particular, a treatment with a combination of an organic solvent (tri-n-butyl phosphate or TnBP) at concentrations between 0.3 and 1%, and detergents such as Triton™ X-100 or Tween® 80, also at concentrations generally between 0.3% and 1%. Such solvent–detergent (S/D) procedures have proven very efficient and robust in the inactivation of lipid-enveloped viruses in human plasma products (*84*). However, use of this method for antivenoms has not been reported.

Implementation of dedicated viral inactivation treatments, such as S/D or other methods, should be encouraged for processes which, based on risk assessment, would offer an insufficient margin of viral safety. Process changes associated with the introduction of new viral reduction steps, and the subsequent removal of any toxic compounds needed for viral inactivation, should be demonstrated not to affect the quality and stability of antivenoms, and most particularly the neutralization efficacy of venoms. Preclinical assessment of the possible impact of newly introduced viral inactivation treatments should be mandatory.

16.4.6 Possible contribution of phenol and cresols

The antibacterial agents, phenol and cresols, and more rarely formaldehyde, are added by most manufacturers to the starting plasma donations as well as to the final liquid antivenom preparations, at a maximum final concentration of 0.25–0.35%. Compounds like phenol are known to be very lipid soluble and lipophilic.

Addition of 0.25% (final concentration) phenol to concentrated antivenom bulk at pH 6.5–6.7, prior to its dilution and formulation, was found to inactivate eight enveloped and non-enveloped viruses very efficiently within 30 minutes (*132*).

Performing additional validations of the virucidal effect of antimicrobial agents as added to the starting hyperimmune plasma and to the final antivenom preparations is encouraged. More information is needed on the potential impact of these antimicrobial agents on the viral safety of antivenoms.

16.5 Production-scale implementation of process steps contributing to viral safety

As there is increasing, although preliminary, evidence that at least some of the existing steps in the production of antivenoms contribute to viral reduction, it is already recommended that specific care should be taken to ensure their appropriate industrial implementation so as not to compromise any possible benefits they provide in terms of viral safety.

Measures should therefore be taken to ensure that such steps are correctly carried out in a manufacturing environment and that cross-contamination and downstream contamination are avoided. Such important aspects of product safety have been highlighted in WHO Guidelines (84) and should also be taken into consideration for large-scale manufacture of antivenoms. Specific attention should be paid to:

- *Process design and layout*: in particular the production floor area needed to carry out such treatment safely, minimizing the chance of cross-contamination between pre-viral-treatment steps product and post-treatment product; the justification for creating a safety zone to avoid risk of downstream contamination, and the procedures used for cleaning and sanitization of the equipment to avoid batch-to-batch cross-contamination.
- *Equipment specifications*: having in mind the potential contribution to viral reduction. For instance, vessels used for low pH incubation or caprylic acid treatment should be fully enclosed and temperature-controlled. There should be no "dead points" where the temperature

defined in the specification or the homogeneity of mixing cannot be ensured. A poor equipment design could compromise the viral safety potentially afforded by a given production step.

- *Qualification and validation*: should verify that the equipment conforms to predefined technical specifications and relevant GMP.
- *Process implementation*: production steps contributing to viral safety such as low pH treatment and caprylic acid treatments could be implemented in two stages performed in two distinct enclosed tanks. Care should be taken to ensure complete process segregation before and after the completion of these treatments to avoid risks of downstream contamination.
- *Process control*: is a critical part of the manufacturing process since completion of viral inactivation and removal cannot be guaranteed by testing the final product. Samples should be taken to confirm that the process conditions of claimed inactivation steps meet the specified limits (for example, for pH, concentration of stabilizers and concentration of virus inactivating agent, such as caprylate). When this is technically feasible and intermediates are stable, samples can be kept frozen for possible additional analysis prior to the release of the batch. It is the responsibility of the manufacturer to ensure that the execution of steps contributing to virus inactivation and removal in a production setting conforms to the conditions that contribute to such virus reduction.
- *SOPs*: steps contributing to viral reduction should be described in approved SOPs. These should contain critical process limits for the viral inactivation and removal methods.
- *Role of the quality assurance department*: because of the critical nature of the viral inactivation and removal steps, quality assurance personnel should review and approve the recorded conditions for viral inactivation and removal while the batch is being processed; that is, not just as part of the final overall review of the batch file.

16.6 Transmissible spongiform encephalopathy

Transmissible spongiform encephalopathy (TSE) has not been identified in any equine species. There has been no case of transmission of TSE linked to antivenoms or other equine-derived blood products.

Of particular concern, however, is that TSEs include scrapie in sheep, a ruminant species that is used, although much less frequently than horses, in the manufacture of antivenoms. Scrapie is a disease similar to bovine spongiform encephalopathy (BSE or "mad cow disease"), but is not known to infect humans.

However, the blood of sheep with experimental BSE or natural scrapie can be infectious and, because scrapie and BSE prion agents behave similarly in sheep and goats, the use of the blood of small ruminants in preparing biologicals should either be avoided or the animals should be selected very carefully from sources known to be free of TSEs. The findings of disease-associated proteins in muscle tissue of sheep with scrapie and the recognition of BSE itself in a goat, reinforce the need for manufacturers of biologicals, including antivenoms, to maintain the precautionary safety measures recommended in the WHO guidelines on TSE tissue infectivity (*77*).

According to these recommendations, the use of tissues or body fluids of ruminant origin should be avoided in the preparation of biological and pharmaceutical products. When sheep-derived materials must be used, they should therefore be obtained from sources assessed to have negligible risk from the infectious agent of scrapie. Documented surveillance records should be available. The feed of animals used for production of hyperimmune plasma should be free of ruminant-derived material.

The infectious agent is thought to be a misfolded, abnormal, prion protein (PrPTSE). It is not yet known whether manufacturing processes used to produce antivenoms from sheep plasma include steps that can contribute to the removal of PrPTSE. Experimental prion clearance studies, based on spiking experiments, can be performed to assess the capacity of the process to remove prions. However, there is still uncertainty about the validity of such experimental studies since the biochemical features of PrPTSE in blood and plasma are not known.

16.7 Main recommendations

- **The viral safety of antivenoms results from a combination of measures:**
  - **to ensure satisfactory health status of the animals;**
  - **to reduce the risk of contamination in the starting raw material;**
  - **to ensure the contribution of the manufacturing process towards inactivation and/or removal of viruses; and**
  - **to ensure compliance with GMP along the entire chain of production.**
- **Manufacturing processes should include at least two steps contributing to robust viral reduction. A virus inactivation step that can be easily monitored is usually preferred to other means of viral reduction, such as nonspecific removal.**

- **Manufacturers must evaluate the capacity of their current manufacturing processes (in particular low pH pepsin digestion, caprylic acid treatment, ammonium sulfate or heat precipitation, and possibly other steps) to inactivate or remove viruses and validate them, if necessary. These studies should be done following existing international guidelines and using relevant and/or model viruses that are representative of the viruses that could affect the animals used for the production of the antivenom immunoglobulins.**
- **The removal of antimicrobial agents from the final formulation of antivenoms should be carefully weighed against the likely benefits these agents may have on the viral safety.**
- **Should the viral reduction processes used be found to be insufficient to ensure a margin of safety, the introduction of dedicated viral reduction methods should be considered. The impact of such process changes on product efficacy and safety should be carefully analysed in vitro as well as in preclinical studies before performing clinical evaluations in humans.**
- **Great attention should be paid to the production-scale implementation of all steps contributing to viral safety to ensure a consistent and reproducible batch-to-batch viral reduction and an absence of risks of cross-contamination and downstream recontamination that would jeopardize the viral safety of the product.**
- **When materials originating from sheep must be used for the production of plasma, they should be obtained from sources assessed to have negligible risk from the infectious agent of scrapie.**

17. Quality control of antivenoms

Quality control of the final product is a key element in the quality assurance of antivenoms. Quality control tests should be performed by the manufacturer or under its responsibility before the product is released. In addition, relevant analyses should be performed on any intermediate steps of the manufacturing protocol as part of the in-process quality control system.

The results obtained should meet the specifications approved for each antivenom product or its intermediates, and constitute part of the batch record. For a liquid preparation, some quality control tests, such as the venom-neutralizing efficacy test or the detection of residual reagents used during fractionation, can be performed on the final bulk and may not need to be repeated

on the final bottled product if the processing after the bulk preparation has been validated and shown not to have any impact. Quality control assessment of the final antivenom product includes the tests described below.

17.1 Standard quality assays

17.1.1 Appearance

The appearance of the product (for example, colour and clarity of the liquid, appearance of the powder) should comply with the description in the marketing dossier.

17.1.2 Solubility (freeze-dried preparations)

The time from the addition of solvent to the complete dissolution of freeze-dried antivenom, under gentle mixing, should be determined. Antivenoms should be completely dissolved within 10 minutes at room temperature. The solution should not be cloudy. Shaking of the container should be avoided to prevent the formation of foam.

17.1.3 Extractable volume

The volume of product extractable from the container should be in compliance with that indicated on the label.

17.1.4 Venom-neutralizing efficacy tests

These tests determine the capability of an antivenom to neutralize the lethal effect of the snake venom(s) against which the antivenom is designed. It is first necessary to determine the lethal potency of the venom, using the LD_{50} assay. The exact volume of antivenom required to neutralize venom lethality can then be determined using the antivenom effective dose (ED_{50}) assay.

The outputs of these tests provide globally applicable standard metrics of: venom lethality and antivenom efficacy, which enable internal monitoring and external, independent auditing of antivenom efficacy – thereby preventing the distribution of ineffective antivenom.

Consistent use of outbred strains of mice, of a defined weight range (for example, 18–20 g) that receive a defined challenge dose, is recommended for all the assays. Some producers use other test animals, such as guinea-pigs. While weights will clearly vary between animal species, a series of principles, specified for mice, will still apply to these alternative test animals. It should be borne in mind that there are variations in the susceptibility of different strains of mice to the lethal effect of venoms.

The venom-neutralizing potency tests are used for quality control and preclinical assessment, so protocol details are described in section 19, while ethical issues are discussed in section 4.

17.1.5 Osmolality

Osmolality is used to measure the tonicity of the antivenom solution, and should be at least 240 mOsmol/kg. Determination of osmolality is also an indirect means to determine the quantity of salts or excipients added for formulating the batch.

17.1.6 Identity test

When several types of antivenoms are produced by a single production facility, a system to identify each batch of antivenom should be established for monitoring and auditing purposes. Identity tests may include biological assays as well as physicochemical and immunological tests. Double immunodiffusion assays, confronting the antivenom with the venoms against which the antivenom is designed to act, are often used. In the case of laboratories that use various animal species to raise antivenoms, that is, horses and sheep, an immunological identity test should be used to identify the mammalian species in which the antivenoms are produced.

17.1.7 Protein concentration

The total protein concentration of antivenoms can be determined using a variety of approaches, including:

- the Kjeldahl method to determine nitrogen content;
- several colorimetric procedures; and
- spectrophotometric (280 nm) assays.

The presence of preservatives should be taken into account since they may interfere with some protein determination methods (*133*).

The total protein concentration of antivenoms should preferably not exceed 10 g/dL, since administration of higher amounts of protein may be associated with higher adverse reaction rates, although some jurisdictions have authorized higher concentrations.

17.1.8 Purity and integrity of the immunoglobulin

The purity and integrity of the active substance, that is, intact immunoglobulin or immunoglobulin fragments, should be assessed to identify contaminants and immunoglobulin degradation. Immunoglobulins or their fragments should constitute the great majority of the preparation, ideally greater than 90%. Evidence suggests, however, that although antivenoms may have physicochemical purity > 90% (for example, immunoglobulins or their fragments), immunochemical purity (for example, specificity for the snake venoms they are produced from) can be lower than 40% (*134*). These findings have emphasized the need to incorporate

both physicochemical and immunochemical analyses in the assessment of antivenom purity.

Electrophoretic methods in polyacrylamide gels (SDS-PAGE run under reducing or non-reducing conditions) are suitable for this purpose, since these techniques allow the detection and monitoring of IgG, $F(ab')_2$, Fab, non-immunoglobulin plasma protein contaminants (in particular albumin) and degradation products. The electrophoretic pattern should be compared to that of a reference preparation. A semi-quantification can be performed by calibration of the procedure. Of particular relevance is the assessment of the albumin content, which ideally should not exceed 1% of total protein content. The following approach can serve as a guide in assessing the purity of antivenoms:

- SDS-PAGE under non-reducing conditions – this analysis provides qualitative (or, at best, semi-quantitative) information on the amounts of intact immunoglobulins, digestion products and, importantly, on the presence of high-molecular-mass oligomers (soluble aggregates) and low-molecular-mass contaminants (which are expected in the case of enzymatically digested antivenoms).
- SDS-PAGE under reducing conditions – analysis under these conditions can provide information on the amount of immunoglobulins and their fragments by direct visualization of intact and/or digested immunoglobulin heavy chains.

17.1.9 Molecular-size distribution

The presence of aggregates and other components in antivenoms can be assessed by size-exclusion liquid chromatography (gel filtration) in fast protein liquid chromatography (FPLC) or HPLC systems.

Densitometric analyses of chromatographic profiles allow the quantification of protein aggregates and of the relative abundances of: intact immunoglobulins, divalent immunoglobulin fragments ($F(ab')_2$, monovalent immunoglobulin fragments (Fab) and dimers, as well as low-molecular-mass enzymatic digestion products.

In intact immunoglobulin-based antivenoms this method allows quantitation of albumin, as its molecular mass (~66 kDa) can be resolved from the ~160 kDa peak of intact immunoglobulins.

17.1.10 Test for pyrogen substances

Antivenoms should comply with the rabbit pyrogen test where required by the local regulations. This test is based on intravenous injection of antivenoms in the ear vein of rabbits. The dose of antivenom must be calculated by dividing the threshold pyrogenic dose in rabbits by the endotoxin although other doses might

be used depending on the pharmacopoeia), followed by the measurement of rectal temperature at various time intervals after injection. The detailed procedures are described in various pharmacopoeias. Bacterial lipopolysaccharides can also be detected by the Limulus amoebocyte lysate (LAL) test. The test should be validated for each type of antivenom, since there have been reports of false-positive and false-negative reactions when testing antivenoms and other plasma-derived products. The sensitivity of this LAL test should be correlated with the rabbit pyrogen test, and the endotoxin limits established (*135*). When regulation allows, a validated LAL test is used in place of the rabbit pyrogen test.

17.1.11 Abnormal toxicity test

The abnormal toxicity test (7 day observation of the effects of intraperitoneal injection of 0.2 mL and 0.5 mL antivenom into mice and guinea-pigs, respectively), is still required by some pharmacopoeias and is performed at the stage of product development.

However, because of the very limited quality control value of this assay, it is increasingly being abandoned by most regulatory authorities. Correct implementation of GMP should provide evidence that the product would comply with the test for abnormal toxicity.

17.1.12 Sterility test

Antivenoms should be free of bacteria and fungi, that is, they should be sterile. The sterility test is performed following methodologies specified in various pharmacopoeias such as the European Pharmacopoeia.

Since antivenoms may contain preservatives in their formulation, it is necessary to "neutralize" the preservatives before the samples are added to culture media. This is usually done by filtering a volume of antivenom through a 0.45 μm pore-size membrane, and then filtering through the same membrane a solution that neutralizes the bacteriostatic and fungistatic effects of the preservatives used in antivenom. The membrane is then aseptically removed and cut into two halves. One half is added to trypticase soy broth and the other is added to thioglycolate medium. Control culture flasks are included for each medium. Flasks are incubated at 20–25 °C (trypticase soy broth) or at 30–35 °C (thioglycolate) for 14 days. Culture flasks are examined daily for bacterial or fungal growth. The number of vials tested per batch should be in compliance with local regulations.

17.1.13 Concentration of sodium chloride and other excipients

The concentration of the various excipients or stabilizers added during formulation should be determined using appropriate chemical methods.

17.1.14 Determination of pH

The pH of antivenom should be determined using a potentiometer.

17.1.15 Concentration of preservatives

Phenol concentration should not exceed 2.5 g/L and cresols 3.5 g/L.

Phenol concentration can be determined spectrophotometrically on the basis of the reactivity of phenol with 4-aminoantipyrine, under alkaline conditions (pH 9.0–9.2) in the presence of potassium ferrocyanide as oxidant. Other methods are also available. Phenol and cresols can be determined by HPLC methods.

17.1.16 Chemical agents used in plasma fractionation

The chemical reagents used in the precipitation and purification of antivenoms, such as ammonium sulfate, caprylic acid and others, should be removed from the final product during diafiltration or dialysis. Limits should be established and their residual amount quantified in the final product. Likewise, the elimination of pepsin or papain from the final preparations should be guaranteed, especially for preparations that are maintained in liquid form, to avoid proteolytic activity that may damage the antivenoms.

The determination of the residual amount of agents used in plasma fractionation could be excluded from routine release testing if the process of manufacturing has been validated to eliminate these reagents. The detection of residual reagents can also be performed on the final bulk rather than in the final product.

17.1.17 Residual moisture (freeze-dried preparations)

Residual moisture content can be determined by several methodologies, such as:

- a gravimetric method assessing the loss of weight on heating;
- the Karl-Fischer titration, based on the principle that iodine, together with pyridine, sulfur dioxide and methanol from the reagent react quantitatively with water;
- thermogravimetric methods.

The methodology most commonly recommended is the Karl-Fischer titration. Every manufacturing and quality control laboratory must establish the accepted maximum residual moisture for their antivenom ensuring the stability of the product over its claimed shelf-life. A residual moisture content of less than 3% is usually recommended for most freeze-dried therapeutic biological products.

17.2 Antivenom reference preparations

The use by manufacturers of in-house reference preparations of antivenoms, instead of national or regional standards, is recommended, since venom-neutralizing efficacy, specificity, and purity can only be compared with antivenoms of similar specificity and neutralizing profile. In-house reference antivenom preparations should be obtained from a suitable batch of a product that has been fully characterized and evaluated by the quality control laboratory. For assays not related to venom-neutralizing efficacy or specificity, such as the quantification of proteins, preservatives and excipients, national or regional standards can be used. The preparation of national or regional reference antivenom preparations should be undertaken by relevant national or regional drug control laboratories and regulatory agencies. These also require comprehensive characterization and evaluation by drug control laboratories and appropriate validation prior to acceptance and establishment.

17.3 Main recommendations

- **Quality control of antivenom preparations, both for intermediate and final products, as part of the batch release, must be performed by the manufacturers and results disclosed in the documentation.**
- **Results from the following quality control tests need to be provided by the manufacturer as part of the batch release documentation:**
  - **(a) venom-neutralizing efficacy test against the most relevant venoms to be neutralized;**
  - **(b) identity test;**
  - **(c) protein concentration;**
  - **(d) purity of the active substance;**
  - **(e) content of protein aggregates and non-IgG contaminants;**
  - **(f) pyrogen test;**
  - **(g) sterility test;**
  - **(h) concentration of excipients;**
  - **(i) osmolality;**
  - **(j) pH;**
  - **(k) concentration of preservatives;**
  - **(l) determination of traces of agents used in plasma fractionation;**
  - **(m) appearance, and, for freeze-dried preparations, residual moisture and solubility; and**
  - **(n) labelling validation and confirmation.**

- **Antivenom reference preparations reflecting specific characteristics of antivenoms produced should be prepared by each manufacturer to be used as standards in their laboratory settings, in particular to measure neutralization capacity of their specific antivenom products against targeted venoms. Relevant standards are also used to establish conformity of purity and integrity. When possible, a national reference antivenom should be established.**
- **It is the ethical responsibility of the manufacturer to use only the minimum number of experimental animals to measure the efficacy of an antivenom.**
- **The development of in vitro methods validated for replacing animal experiments is strongly encouraged.**

18. Stability, storage and distribution of antivenoms

18.1 Stability

Stability studies should be performed to determine the stability of antivenoms. These studies should be done when a new product, a process change, or a new formulation is developed. They are essential to define the shelf-life of the product and are intended to prove that the antivenom remains stable and efficacious until the expiry date. During the developmental stages, stability studies should be included. Their design should take into consideration that they are a complex set of procedures involving considerable cost, time and expertise necessary to build in quality, efficacy and safety of a product.

Most liquid antivenom preparations have a shelf-life of up to 3 years when stored refrigerated at 2–8 °C, whereas freeze-dried antivenoms have shelf-lives of up to 5 years when kept in the dark at room temperature. It is essential, however, that manufacturers determine the actual stability of each antivenom formulation under appropriate conditions using validated methodologies. It is also highly recommended that manufacturers perform stability studies to evaluate the possibility that their preparations could be stored for a long period without refrigeration (for instance at 30 °C).

Real-time stability tests should be performed under the expected storage conditions of the antivenom. In addition, these tests could be performed under worst-case storage conditions. Quality control parameters are determined at regular pre-established time intervals, normally extending the test period in order to allow significant product degradation under recommended storage conditions. Essential parameters include venom neutralization potency, turbidity and content of aggregates, among others, since these are especially prone to alter upon storage.

Accelerated stability studies may be performed to provide early useful information on the product stability profile, but are not a substitute for real-time data. In accelerated studies, the antivenom is exposed to harsher conditions than usual, such as a higher temperature, and the stability is assessed over a shorter time span. This is done to assess the conditions that accelerate degradation of the product and this information is then used to predict shelf-life.

Retained sample stability testing is usual practice for every product for which stability data are required. In such a study, retained samples from at least one batch a year are selected and tested at predetermined intervals – that is, if a product has shelf-life of 5 years, it is conventional to test samples at 3, 6, 9, 12, 18, 24, 36, 48, and 60 months.

Cyclic temperature stress testing is not routinely performed, but it may be useful since it is designed to mimic likely conditions in field place storage. It is recommended that the minimum and maximum temperatures are selected on a product-by-product basis and taking into account factors like recommended storage temperatures as well as specific chemical and physical degradation properties.

18.2 Storage

Antivenoms should be stored at a temperature within the range that assures stability, as found by stability tests. This is particularly critical for liquid formulations, which usually require storage at between 2 and 8 °C. Therefore, deviations from this temperature range, due to interruptions in the cold chain during transportation or storage, are likely to result in product deterioration. The design of adequate cold chain programmes, as part of the public health systems in every country, is critical, and national protocols should be developed. The distribution policies for national vaccination programmes can be adopted for the transportation and storage of antivenoms. The stability of liquid preparations at temperatures higher than 2–8 °C should be evaluated and, if needed, new formulations allowing such storage conditions should be developed.

18.3 Distribution

Adequate distribution of antivenoms is a matter of great concern in many regions of the world. Since most of the antivenoms available are liquid preparations, the maintenance of an adequate cold chain must be guaranteed, despite the difficulties to be encountered in rural areas of some developing countries. National and regional health authorities should develop distribution strategies to ensure that antivenoms are allocated to the areas where they are needed or use the distribution channels in place for other national primary health-care programmes. Both the specificity of the antivenom and the number

of vials or ampoules to be distributed should be taken into consideration. This is particularly relevant in countries that use monospecific antivenoms, since distribution of these products should be guided by the known distribution of the species and the epidemiological data. To ensure an appropriate supply for clinical use, inventories should be in excess of the estimated number of cases, to allow for unpredictable surges in local demand, accepting that some antivenoms will not have been used before their expiry date.

18.4 Main recommendations

- **The quality control of each antivenom batch prepared by a manufacturer should include the potency test for neutralization of lethality (ED_{50}).**
- **In general, liquid preparations require a cold chain, whereas freeze-dried preparations do not. However, storage conditions are product- or formulation-specific and may vary. Manufacturers should therefore determine the stability of each antivenom pharmaceutical preparation by conducting real-time stability studies.**
- **Manufacturers should study the stability of antivenoms at the ambient temperatures in the areas where the product will be used.**
- **The distribution of antivenoms by health authorities should rely on a proper assessment of the epidemiology of snake-bite envenomings, and on the proper knowledge of the geographical distribution of the most relevant venomous species. This is particularly important for monospecific antivenoms.**
- **NRAs should ask manufacturers to provide information obtained from the preclinical assessment of all antivenom used in their territories against the venoms found in the region or country where the product is intended to be used.**

19. Preclinical assessment of antivenom efficacy

Efficacy testing of antivenoms is one of a suite of assessments required for the quality control of antivenoms (see section 17 where further quality control tests are described) performed for each new batch. Efficacy testing of antivenoms is also part of the preclinical programme to be performed for new antivenoms, where the respective data are used for licensing or registration of antivenoms by regulatory agencies. The details of efficacy testing in the preclinical phase of antivenoms and for quality control purposes are described below. The testing of antivenoms on animals raises important ethical considerations (section 4) and it is essential that manufacturers and others apply the highest standards of ethical

conduct, including appropriate 3R steps, and use of analgesia or anaesthesia for the minimization of pain and discomfort.

It is a fundamental regulatory and ethical requirement that all new therapeutic agents for human use are tested for their safety and efficacy – initially by in vitro LD_{50} laboratory tests and then in vivo ED_{50} preclinical tests and, if the results of these prove satisfactory, by clinical trials in human patients. The preclinical efficacy tests must therefore be performed on new antivenoms, and newly manufactured batches of existing antivenoms.

19.1 Preliminary steps that may limit the need for animal experimentation

To prevent unnecessary animal use, careful perusal of existing literature for data on venom lethality may help to refine the experimental design and thereby reduce the number of experimental animals required.

Manufacturers may also investigate the immunological venom-binding capability of an antivenom by performing immunological assays (for example, ELISA, to identify, and exclude from experimentation, antivenoms that do not possess the requisite titre of venom-binding immunoglobulins. It is crucial to note, however, that: (a) a high venom-binding titre in an ELISA result for an antivenom cannot be used to infer venom-neutralizing efficacy; and (b) the failure of an antivenom to bind venom in an ELISA result suggests very strongly that the antivenom should be considered ineffective at neutralizing the effects of that venom – and withdrawn from ED_{50} testing. This step can further limit non-productive animal experimentation. There is no single ELISA metric that enables stop/go decisions to be made for all the possible snake venom and antivenom combinations. These will therefore be in-house decisions.

An additional immunological cross-reactivity technology that can inform the preclinical assessment process before animal experiments are undertaken is the use of a proteomics-centred platform, termed antivenomics, which has been developed to assess the immunological reactivity of antivenoms against homologous and heterologous venoms (*136–139*). Antivenomics complements the in vitro and in vivo venom activity neutralization assays and substitute for the traditional, essentially qualitative, immunological methods, such as ELISA and Western blotting. Antivenomics uses an affinity chromatography approach to investigate the immuno-capturing ability of immobilized IgG, $F(ab')_2$, or Fab antibody molecules followed by the proteomic identification of the venom components recovered in both the retained and the non-bound fractions. The fraction of non-immuno-captured protein "i" (%NRi) is estimated as the relative ratio of the chromatographic areas of the same protein recovered in the non-retained (NRi) and retained (Ri) affinity chromatography fractions using the equation:

$$\%NRi = 100 - [(Ri/(Ri + NRi)) \times 100]$$

The antivenomic analysis provides both qualitative and quantitative information on the types of venom proteins presenting antivenom-recognized epitopes and those exhibiting impaired immunoreactivity. Although the level of immune recognition gathered from antivenomics should not be absolutely relied upon to predict the in vivo neutralization capacity of an antivenom (since both experiments involve radically different protocols), an immuno-capture capability of ≥ 25% generally correlates with a good outcome in homologous in vivo neutralization tests. If immuno-capture by this method is < 25% the further testing of an antivenom using in vivo methods should be reconsidered.

As the degree of immuno-recognition of a given toxin by the immunoglobulins present in antivenom represents a measure of the capability of that particular antivenom to neutralize the toxic activity of that toxin, the antivenomics analysis may assist in assessing the range of clinical applications of current commercial or experimental antivenoms, and in the development of improved antivenoms on an immunologically sound basis. Growing evidence shows the potential of the combination of antivenomics and neutralization assays for analysing at the molecular level the preclinical efficacy of antivenoms against homologous and heterologous venoms. This is particularly so where antivenoms from more than one manufacturer, or from more than one batch of antivenom produced by a manufacturer require preclinical evaluation against the same venoms (*136*, *140*).

Manufacturers should take steps to incorporate these approaches into their preliminary screening of antivenoms before in vivo animal testing is conducted.

19.2 Essential preclinical assays to measure antivenom neutralization of venom-induced lethality

These tests determine the capability of an antivenom to neutralize the lethal effect of the snake venom(s). It is first necessary to determine the lethal potency of the venom using the LD_{50} assay. The exact volume of antivenom, or the venom/antivenom ratio, required to neutralize venom lethality can then be determined using the antivenom effective dose (ED_{50}) assay.

Preclinical testing of antivenom is required:

- for routine quality control of efficacy of each newly manufactured batch of an existing antivenom;
- to test the ability of a new antivenom to neutralize the lethal effects of venoms from snakes from the country or region where it is going to be introduced;

- to test the ability of an existing antivenom to neutralize the lethal effects of venoms for which no prior ED_{50} data exist (for example, prior to introducing an antivenom to treat envenoming in a new geographical region or country).

The outputs of these tests provide globally applicable standard metrics of venom lethality and antivenom efficacy. This enables internal/manufacturer monitoring and external/independent auditing of antivenom efficacy – thereby providing manufacturers and NRAs worldwide with a standardized mechanism for preventing the distribution of dangerously ineffective antivenom.

Consistent use of venom standards and outbred strains of mice of a defined weight range (for example, 18–20 g), are recommended for all the assays. Some producers use other test animals, such as guinea-pigs. While weights will clearly vary between animal species, the following principles, specified for mice, will still apply to these alternative test animals. It should be borne in mind that there are variations in the susceptibility of various strains of mice to the lethal effect of venoms.

19.2.1 LD_{50} range-finding test

For venoms whose LD_{50} is unknown, it is recommended that a range dose-finding study, using one mouse per venom dose, is performed to set a narrow range of dose parameters for the full LD_{50} test – reducing the total number of animals required. Range-finding tests are not required for venoms from validated suppliers where the lethal potency across successive batches of venom is established. If venom is sourced from a new supplier, re-establishment of LD_{50} should be performed.

Various venom doses are prepared using saline as diluent, and aliquots of a precise volume (maximum 0.2 mL) of each dose are injected, using one mouse per dose, by the intravenous route, in the tail vein (or, alternatively, by the intraperitoneal route (using injection volumes of maximum 0.5 mL)). Deaths are recorded at 24 hours (intravenous test) or at 48 hours (intraperitoneal test). On the basis of this preliminary dose-finding experiment, a range of venom doses causing 0% to 100% lethality is established and thus narrows the range of venom doses required for the full venom LD_{50} assay.

19.2.2 The LD_{50} assay

Venom doses are prepared in saline and intravenously injected (maximum 0.2 mL) into the tail vein of groups of 5–6 mice (of a defined weight range). A group size of five mice is the smallest number recommended for obtaining a statistically significant result. In some laboratories the LD_{50} is estimated by the intraperitoneal route using an injection volume of a maximum of 0.5 mL.

Deaths are recorded at 24 hours (for assays involving intravenous injections) or at 48 hours (when intraperitoneal injections are used), and LD_{50} is estimated by Probit analysis (*141*), Spearman-Karber (*11*) or alternative procedures (such as non-parametric methods). One venom LD_{50} dose is defined as the amount of venom causing death in 50% of injected mice.

19.2.3 Antivenom efficacy assessment

The venom LD_{50} results provide the information necessary to test the venom-neutralizing efficacy of an antivenom – using the median effective dose (ED_{50}) assay. It is important that the venoms used in the ED_{50} assays are from the same batch (lot) as that used to determine the venom LD_{50} result. It is equally important that all the LD_{50} and ED_{50} assays utilize mice of identical strain and weight.

19.2.3.1 ED_{50} range-finding test

For an antivenom whose ED_{50} against a specific venom is unknown, it is recommended that a range dose-finding study, using one mouse per venom dose, is performed to set a narrow range of dose parameters for the full ED_{50} test – reducing the total number of animals required. Range-finding tests are not required for an antivenom whose venom-neutralizing efficacy is established.

The selected multiple of the venom LD_{50} (3–6 LD_{50}) is mixed with different doses of antivenom, incubated at 37 °C for 30 minutes, and each mixture then intravenously injected into a single mouse. This preliminary test establishes a range of antivenom volumes that result in 100% survival and 100% death of the injected mice and thus narrows the range of doses required for the formal antivenom ED_{50} test.

19.2.3.2 The median effective dose (ED_{50}) assay

A fixed amount of venom ("challenge dose", usually corresponding to 3–6 LD_{50}) is mixed with various volumes of the antivenom and adjusted to a constant final volume with saline (*3*, *142*, *143*). The mixtures are incubated for 30 minutes at 37 °C, and then aliquots of a precise volume (maximum 0.2 mL intravenously; maximum 0.5 mL intraperitoneally) of each mixture are injected into groups of 5–6 mice[10] (of the same strain and weight range used for the LD_{50} assay). A control group injected with a mixture of the venom "challenge dose" with saline solution alone (no antivenom) should be included to confirm that the venom "challenge dose" induces 100% lethality. Centrifugation of the antivenom–venom

[10] Ten mice may be needed for some venoms.

mixtures before use is not recommended because residual venom toxicity may remain in the immuno-precipitate.

After injection, deaths are recorded at 24 hours (intravenous test) or at 48 hours (intraperitoneal test) and the results analysed using Probit analysis, Spearman-Karber or alternative procedures (such as non-parametric methods). One antivenom ED_{50} dose is defined as the amount of antivenom, or the venom/antivenom ratio, resulting in the survival of 50% of mice injected with a mixture of antivenom and a lethal quantity of venom.

The ED_{50} result can be expressed in various ways:

- mg of venom neutralized by mL of antivenom;
- µL antivenom required to neutralize the "challenge dose" of venom used;
- µL of antivenom required to neutralize 1 mg of venom.

The practice of defining the ED_{50} by the number of murine LD_{50}s of venom neutralized per mL of antivenom is inaccurate and has little clinical usefulness. Since LD_{50} values for the same venom may vary from one manufacturer to another, this representation of ED_{50} should be avoided in favour of one of the approaches listed above.

19.2.4 General recommendations

Before any antivenom is used therapeutically in humans, its efficacy against the relevant snake venoms should be confirmed in the essential preclinical LD_{50} and ED_{50} assays. Where minimum standards for venom-neutralizing efficacy exist in geographically relevant pharmacopoeias, or have been established by NRAs, these requirements must be met.

In some regions, no minimum acceptable levels of therapeutic efficacy that are clinically relevant to human envenoming have been established that take into account the need to deliver a therapeutic dose of antivenom in a realistic volume for administration. In such cases, NRAs in consultation with other organizations should establish such standards as a matter of priority for the various antivenoms produced or distributed in these jurisdictions.

The essential tests of preclinical efficacy, the venom LD_{50} and antivenom ED_{50}, should be standardized by NRAs and national quality control laboratories, and common protocols adopted to avoid variation in methodology between production facilities. Therefore, manufacturers should disclose details of their ED_{50} protocol to the corresponding NRA as part of the licensing or registration application to demonstrate compliance.

Quality control laboratories need to establish national reference venom collections (venoms representing the taxonomic and geographical range of

snake species in a country), and these must periodically be independently evaluated to ensure that they have not deteriorated (see section 10 on quality control of venoms).

19.3 Supplementary preclinical assays to measure antivenom neutralization of specific venom-induced pathologies

Snake venoms generate a wide range of systemic pathologies, including a variety of haemostasis-disruptive (haemorrhage, pro- and anti-coagulopathic effects), neurotoxic, myotoxic, nephrotoxic and cardiac effects. Supplementary tests are therefore recommended for new antivenoms and for new applications of existing antivenoms to determine whether they are effective in eliminating the most clinically relevant pathophysiological effects induced by the specific venom(s) of interest.

For example, a new antivenom developed against Echis ocellatus envenoming should, in addition to preclinical LD_{50} and ED_{50} testing, be tested for its ability to eliminate venom-induced coagulopathy and haemorrhage – the most medically important effects of envenoming by *E. ocellatus*.

In this context it may be useful to consider that postmortem observations of mice from LD_{50} and associated ED_{50} experiments can provide a wealth of pathophysiological information as to antivenom neutralization of venom-induced pathology. Postmortem observations from LD_{50} and associated ED_{50} experiments may prove useful in reducing the need for and frequency of some of the supplementary assays recommended here; however, their use requires the same degree of scientific and procedural validation as other supplementary preclinical assays.

These supplementary preclinical tests are outlined below.

19.3.1 Neutralization of venom haemorrhagic activity

Many venoms, especially those of vipers, exert powerful local and systemic haemorrhagic activity effected primarily by snake venom zinc-dependent metalloproteinases. These enzymes damage the basement membrane that surrounds the endothelial cells of capillaries resulting in bleeding into the tissues. Bleeding into the brain and other major organs is considered to be the major lethal effect of envenoming by many viperid species (*144*). The minimum haemorrhagic dose of a venom (MHD) quantifies this venom-induced pathology, and is defined as the amount of venom (in µg dry weight) which, when injected intradermally, induces in mice a 10 mm haemorrhagic lesion after a predefined time interval, usually 2–3 hours, after injection (*145*, *146*).

The venom MHD test is carried out by preparing aliquots of 50 µL of physiological saline solution containing a range of venom doses. Mice (18–20 g

body weight; five mice per group) are held securely and the hair surrounding the injection site is shaved. Venom solutions (50 µL) are injected intradermally into the shaved skin of lightly anaesthetized mice. After a defined time interval (usually 2–3 hours), mice are killed using an approved humane procedure, the area of the injected skin is removed, and the size of the haemorrhagic lesion in the inner side of the skin is measured using calipers in two directions with background illumination. Care should be taken not to stretch the skin. The mean diameter of the haemorrhagic lesion is calculated for each venom dose and the MHD estimated by plotting mean lesion diameter against venom dose and reading off the dose corresponding to a 10 mm diameter (*145*, *146*).

The assay measuring the efficacy of antivenom to neutralize venom-induced haemorrhage is termed the MHD-median effective dose (MHD_{50}), and is defined as the volume of antivenom, in microlitres, or the venom/antivenom ratio, which reduces the diameter of haemorrhagic lesions by 50% when compared with the diameter of the lesion in animals injected with the control venom/saline mixture (*146*). A "challenge dose" of venom is selected – between one and five venom MHDs have been used as the challenge dose by different laboratories. The test is carried out as above, using five mice per group. Mixtures of a fixed amount of venom and various dilutions of antivenom are prepared so that the challenge dose of venom is contained in 50 µL. Controls must include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are injected intradermally in lightly anaesthetized mice. The diameter of haemorrhagic lesions is quantified as described above, and the neutralizing ability of antivenom is expressed as the MHD_{50}.

19.3.2 Neutralization of venom necrotizing activity

Venom-induced local dermonecrosis is a major problem in humans bitten by snakes and it has long been considered important to have an assay system to evaluate the effect of an antivenom on this pathology. However, the value of antivenoms in overcoming the cytolytic effects of venoms has not yet been established; indeed, there is considerable doubt as to whether antivenom is useful in obviating such effects in human victims of snake-bite. This is because venom-induced dermonecrosis occurs quickly after a bite and there is usually a considerable delay between the envenoming of a victim and his or her arrival in hospital for treatment. Consequently, antivenom therapy can have little or no effect in reversing the damage (*147*, *148*). Animal experiments in which the antivenom was administered to the test animal at different times after the venom support this opinion (*148–150*). The minimum necrotizing dose (MND) of a venom is defined as the smallest amount of venom (in µg dry weight) which, when injected intradermally into groups of five lightly anaesthetized

mice (18–20 g body weight), results in a necrotic lesion of 5 mm diameter 3 days later. The method used is the same as that for the MHD, except that the skin is examined 3 days after the intradermal injection of the venom (*145*).

The assay measuring the ability of an antivenom to neutralize venom-induced dermonecrosis is termed the MND-median effective dose (MND_{50}), and is defined as the volume of antivenom, in microlitres or the venom/antivenom ratio, which reduces the diameter of necrotic lesions by 50% when compared with the diameter of the lesion in mice injected with the control venom/saline mixture. A challenge dose of venom is selected, usually between one and two MNDs. The test is carried out as above, using five mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the venom challenge dose is contained in 50 µL. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are injected intradermally in lightly anaesthetized mice (*151*, *152*). The diameter of dermonecrotic lesions is quantified 3 days post-injection, as described above, and the neutralization by antivenom, expressed as the MND_{50}.

19.3.3 Neutralization of venom procoagulant effect

Many venoms, especially from some vipers, cause consumption of coagulation factors, which results in incoagulable blood. This, combined with the haemorrhagic nature of some of these venoms, can result in a very poor prognosis for severely envenomed patients. Simple in vitro methods exist to measure this venom-induced pathophysiological effect and the ability of an antivenom to eliminate it. The minimum coagulant dose (MCD) of a venom is defined as the smallest amount of venom (in mg dry weight per litre of test solution or µg/mL) that clots either a solution of bovine fibrinogen (2.0 g/L) in 60 seconds at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (fibrinogen content 2.8 g/L) under the same conditions (MCD-P).

For measurement of the MCD-F, 50 µL of physiological saline with final venom concentrations ranging from 240 to 0.5 mg/L is added to 0.2 mL of bovine fibrinogen solution (2.0 g/L) at 37 °C in new glass clotting tubes. The solutions are mixed thoroughly and the clotting time recorded. The MCD-P is estimated by adding the same venom concentrations to 0.2 mL of the standard citrated human plasma solution under identical conditions and recording the clotting time. In each case, the MCD is calculated by plotting clotting time against venom concentration and reading off the level at the 60 second clotting time (*145*).

To estimate the ability of an antivenom to neutralize venom procoagulant activity, a challenge dose of venom is selected, which corresponds to one MCD-P or one MCD-F. Mixtures of a fixed concentration of venom and various dilutions

of antivenom are prepared so that the challenge dose of venom is contained in 50 µL. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are added to 0.2 mL of plasma or fibrinogen solution, as described. The formation or absence of clots is observed during a maximum period of 30 minutes. The minimum volume of antivenom, or venom/antivenom ratio that completely prevents clotting is estimated and is known either as the MCD-F-effective dose ($MCD\text{-}F_{100}$) or MCD-P-effective dose ($MCD\text{-}P_{100}$).

19.3.4 Neutralization of in vivo venom defibrinogenating activity

This test is a direct measure of the in vivo defibrinogenating effect of certain venoms. To measure the minimum venom defibrinogenating dose (MDD), a wide range of venom doses is selected and each dose, in a volume of 0.2 mL, is injected intravenously into five mice (18–20 g body weight). One hour after injection, the mice are placed under terminal general anaesthesia and bled by cardiac puncture. The blood from each animal is placed in a new glass clotting tube, left at room temperature for 1 hour and the presence or absence of a clot recorded by gently tilting the tube. The MDD is defined as the minimum dose of venom that produces incoagulable blood in all mice tested within 1 hour of intravenous injection.

Antivenom neutralization of the venom component(s) responsible for in vivo defibrinogenation is estimated by incubating a challenge dose of venom, corresponding to one or more MDD, with different dilutions of the antivenom. Controls should include venom solutions incubated with saline solution instead of antivenom. Mixtures are incubated at 37 °C for 30 minutes before injection of 0.2 mL by the intravenous route in groups of five mice (18–20 g body weight). After 1 hour, mice are bled as described above, and the blood is placed in new glass clotting tubes and left undisturbed for 1 hour at room temperature, after which the presence or absence of a clot is recorded. Neutralizing ability of antivenoms is expressed as MDD-effective dose (MDD_{100}), corresponding to the minimum volume of antivenom, or venom/antivenom ratio at which the blood samples of all injected mice showed clot formation (*152*, *153*).

19.3.5 Neutralization of venom myotoxic activity

The presence of myotoxic components in a venom results in the degeneration of skeletal muscle by myonecrosis of muscle fibres. Damage is characterized by the disruption of the muscle cell plasma membranes, myofilament hypercontraction, local infiltration of inflammatory cells and oedema. Myotoxicity is characterized by the appearance of myoglobin in urine and by increments in the serum levels of muscle-derived enzymes, such as creatine kinase (CK). Myotoxic phospholipase A2 (PLA2) enzymes are found in a wide range of snake venoms. Some of these

PLA2s may be primarily myotoxic, or neurotoxic or both. Cytotoxic proteins of the three-finger toxin family present in some elapid venoms also cause myonecrosis. In addition, myotoxicity may occur as a consequence of ischaemia induced in muscle fibres by the effect of haemorrhagic venom components in the microvasculature (*154*).

Venom myotoxic activity is determined by injecting mice with various doses of venom in a constant volume of 50 µL (using saline solution as diluent) into the right gastrocnemius muscle. Groups of five animals of 18–20 g body weight are used per dose. Control animals are injected with the same volume of saline solution. Tail-snip blood samples are collected after a specified time interval (3 hours in mice), and the CK activity of serum or plasma is determined using commercially available diagnostic kits (*155*, *156*). Myotoxic activity is expressed as the minimum myotoxic dose (MMD), defined as the amount of venom that induces an increment in serum or plasma CK activity corresponding to four times the activity in serum or plasma of animals injected with saline solution alone. Myotoxicity can also be assessed by histological evaluation of muscle damage after venom injection, although this is a more expensive and more time consuming method than the CK determination.

To estimate the ability of an antivenom to neutralize venom myotoxicity, a challenge dose of venom is selected, which corresponds to 3 MMDs. The test is carried out as above, using five mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the challenge dose of venom is contained in 50 µL. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 minutes, and aliquots of 50 µL are injected into the gastrocnemius muscle, as described above. Blood samples are collected 3 hours after injection (in the case of mice) and serum or plasma CK activity is quantified. The neutralizing ability of antivenom, expressed as MMD-median effective dose (MMD_{50}) is estimated as the volume of antivenom in microlitres, or the venom/antivenom ratio, which reduces the serum or plasma CK activity by 50% when compared to the activity in animals injected with venom incubated with saline solution only (*143*).

19.3.6 Neutralization of venom neurotoxic activity

Several laboratory methods for assessing venom-induced neurotoxicity have been developed (for example, chick biventer cervicis nerve-muscle preparation (*157*, *158*) and the mouse hemidiaphragm phrenic nerve preparation (*2*, *159–162*). However, they are difficult to perform, require costly equipment and technological expertise and are unlikely to be practicable for most antivenom producers. Mouse lethality tests are usually reliable in predicting the neutralization of neurotoxic effects of venoms.

19.4 Limitations of preclinical assays

It is acknowledged that the in vivo and in vitro essential and supplementary preclinical tests have physiological limitations. Venom and venom/antivenom injection protocols do not represent the natural situation, and the physiological responses of rodents to envenoming and treatment may differ from those of humans. Even comparing the levels of immune recognition gathered from antivenomic or ELISA data with the in vivo neutralization capacity of an antivenom, is not straightforward. Such limitations make the rodent model of human envenoming and treatment less than ideal. Care should therefore be taken to avoid simplistic extrapolations from this assay to the clinical situation. Nevertheless, the LD_{50} and ED_{50} tests represent the methods most widely used for assessment of antivenom potency, and a number of clinical trials have demonstrated that the ED_{50} test is useful (*2*, *163*), but not infallible (*164*, *165*), at predicting the efficacy of antivenoms in the clinical setting. In some cases, it is recommended to test the ability of antivenoms to neutralize pathophysiologically relevant effects other than the lethal effect. Examples include the neutralization of haemorrhagic and in vitro coagulant effects in the case of *Echis* sp. venoms, and of dermonecrotizing effect in the case of cytotoxic *Naja* sp. venoms. An additional value of these tests is the assurance that antivenoms are manufactured with an accepted, quantifiable and uniform neutralizing potency.

19.5 Main recommendations

- **The estimation of the ability of an antivenom to neutralize the lethal activity of venom(s) (LD_{50} and ED_{50}) is a critical preclinical assessment and should be performed by manufacturers for all antivenoms, and enforced by the NRAs as part of the antivenom licensing procedure.**
- **All practitioners of these preclinical tests must prioritize the implementation of 3R to reduce the substantial number of mice used, and their collective pain, harm and distress.**
- **In vitro methods such as ELISA, antivenomics or other emerging technologies that enable antivenoms to be screened for immune recognition of venom components prior to in vivo evaluation should be adopted by manufacturers.**
- **The development of improved in vivo assay protocols to reduce pain and suffering of animals, such as routine use of opioids or other analgesics, and of in vitro alternatives to the in vivo assays to reduce the number of animals used in preclinical testing, is encouraged. The results of any modified in vivo, or new in vitro**

protocols, should be rigorously compared with results from existing protocols and validated to ensure statistical reliability of the newly developed methods.

- **All new antivenoms, as well as existing antivenoms to be used in new geographical areas, should furthermore be assessed for their ability to eliminate specific pathologies caused by the venoms of the snakes for which the antivenom has been designed.**
- **The selection of which preclinical supplementary test(s) to perform will depend on the predominant pathophysiological effects induced by the specific snake venom and be appropriately adapted for each antivenom. These supplementary tests are not required for quality control assessment of subsequent batches of antivenom.**

20. Clinical assessment of antivenoms

20.1 Introduction

Antivenoms are unusual among pharmaceutical agents in that they have been used in human patients since 1896 with little attention being paid to clinical trials of their effectiveness and safety. However, since the 1970s it has been clearly demonstrated that it is possible to carry out dose-finding and randomized controlled trials (RCTs) in human victims of snake-bite envenoming. These studies have yielded invaluable information, as in the case of clinical trials of other therapeutic agents for which clinical trials are generally regarded as the essential basis for regulatory approval.

The conduct of clinical studies is guided by the principles set down in the international regulations governing good clinical practice,[11] comprising European Union, United Kingdom and USA regulations, summarized in ICH Topic E 6 (R1) Guideline for good clinical practice (GCP), an international ethical and scientific quality standard for designing, conducting, recording and reporting trials that involve the participation of human subjects.[12] These principles emphasize the responsibilities of the researcher and of the organization sponsoring the research to protect participants in the research and to ensure that the conduct of the trial is likely to lead to reliable results.

[11] See http://www.therqa.com/committees-working-parties/good-clinical-practice/regulations-and-guidelines/, accessed 23 April 2017.

[12] http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002874.pdf, accessed 23 April 2017.

Clinical trials should be registered with an appropriate registration body, before they commence.[13]

The conventional pathway for clinical evaluation of new therapeutic products is:

- Phase I: healthy volunteer studies – detection of unanticipated adverse events;
- Phase II: limited effectiveness and safety studies, often dose-finding;
- Phase III: full-scale clinical evaluation, often using blinded RCTs to avoid potential introduction of bias;
- Phase IV: post-marketing surveillance.

The appropriateness of this pathway for antivenoms depends upon a number of factors, including whether an antivenom is new or has been previously used in human patients, the ethical basis for the study, the trial's practicability as well as ethical and national regulatory considerations. So far, most antivenoms have been registered without prior clinical studies. This situation should not persist: it is desirable, first, to collect the existing clinical data on antivenoms already marketed, and second to promote Phase II or III clinical trials before registering new antivenoms. In the absence of clinical data for antivenoms already in use, appropriate clinical trials should be quickly implemented.

20.1.1 Identification of biting species in clinical studies of antivenoms

It is absolutely essential that all clinical studies of antivenom effectiveness or safety, including clinical trials incorporate robust methodologies for ensuring the identification of the biting species. This can be achieved through:

- expert identification of the dead snake responsible for the bite, or of a photographic image of that snake; or
- the identification of specific venom components unique to particular species (from bite site swabs, wound exudate, serum or urine samples) through the use of EIAs (ELISA) or other immunological methods.

Failure to properly identify the species of snakes that are responsible for cases of envenoming included in clinical trials and other studies of snake antivenoms significantly diminishes the value of the research, and renders the

[13] https://clinicaltrials.gov/ct2/manage-recs/how-register; http://www.isrctn.com/; https://www.clinicaltrialsregister.eu/; http://www.umin.ac.jp/ctr/; http://www.anzctr.org.au/

results unreliable. NRAs should be cautious about accepting the results of clinical evaluations of antivenom where robust, reliable identification of the biting species is not available.

20.1.2 Phase I studies

Conventional clinical studies using healthy volunteers are not appropriate in the case of antivenoms[14] because of the risk of anaphylactic and other reactions (for example, pyrogenic or serum sickness and, rarely, hypersensitivity reactions to equine or ovine plasma proteins) and the risk of sensitization to equine or ovine plasma proteins in the volunteers. Phase I studies are primarily designed to detect unanticipated adverse reactions. This can be done only in human subjects as it is not possible in an animal model. Such studies are an essential protection against severe and even fatal effects of a new medication, before it is tested in the much larger numbers of subjects demanded for Phase II and III studies. Recent disasters or near disasters during Phase I studies of new therapeutic monoclonal antibodies have emphasized not only the need for such studies but also their potential dangers. A similar situation exists in the early testing of cytotoxic drugs and antibodies used in oncology. A preliminary open-label dose-finding study can establish both the effectiveness and safety of an initial dose of a new antivenom in small groups of adult, non-pregnant patients with systemic envenoming, but excluding those with features of severe envenoming. The aim is to assess clinical safety and effectiveness, as a prelude to full-scale Phase II or III RCTs. This modified Phase I approach can be combined with a preliminary dose-finding study. The "3 + 3" dose-escalation design (166) can be used to determine the minimum dose capable of achieving a defined end-point in two-thirds or more of a small group of patients. An additional stopping rule is added to ensure that patients are not exposed to doses likely to cause reactions in more than one-third of them – for details see Abubakar et al., 2010 (*94*). Currently, there is no alternative for ethical Phase I studies.

20.1.3 Phase II and III studies

Phase II studies are usually conducted to optimize doses, establish or confirm the relative safety of a product and give an indication of effectiveness. Phase III studies are normally used to establish effectiveness of a product, often in comparison with an existing product, or occasionally a placebo. Since antivenoms are so well established in the treatment of snake-bite envenoming, the use of placebo controls is ethically acceptable only where there is genuine uncertainty

[14] Immunoglobulins derived from animal plasma.

about whether the benefit (degree of clinical improvement) from the antivenom outweighs the risk (potential incidence and severity of adverse events). Depending on the speed of evolution of envenoming, immediate treatment might be compared to delayed antivenom treatment. A new antivenom with demonstrable preclinical potency (see above) can be compared with an established product, or two markedly different initial doses or regimens of the same antivenom can be compared. In RCTs, non-inferiority, rather than superiority of a new antivenom or regimen, compared to an existing treatment, requires smaller numbers of trial participants to achieve acceptable power (*94*). Basic requirements for any clinical antivenom RCT are that the participants should be reasonably homogeneous as far as the species of snake responsible and their pretreatment characteristics (for example, interval between bite and treatment) are concerned, and that objective clinical end-points should be selected to judge effectiveness, and measure rates of adverse events.

20.1.4 Phase IV studies

Phase IV studies are clinical surveillance studies that occur after market authorization of the product. In view of the difficulty in performing standard clinical trials of antivenom in some situations, this may be the only way to study safety and effectiveness of an antivenom in a large number of patients. In practice, such studies have rarely if ever been attempted for antivenoms, but they are strongly recommended for the future.

20.2 Clinical studies of antivenom

Although preclinical testing may be valuable in ensuring that antivenoms neutralize the venoms of interest, the complex effects of venoms in humans and the need to consider venom pharmacokinetics mean that, ultimately, the effectiveness and safety of antivenoms for the treatment of human envenoming can only be determined by well-designed clinical studies. Clinical studies of antivenoms primarily address three main issues:

- assessment of the optimal initial dose of antivenom;
- assessment of effectiveness of the antivenom;
- assessment of the safety of an antivenom, particularly the incidence and severity of early and late reactions.

Antivenom safety and tolerance depend on manufacturing factors (immunoglobulins composition, purification of immunoglobulin fragments, protein concentration, and presence of preservatives) (*167*). Consequently, incidence and severity of adverse reactions to similar doses of a given batch of antivenom are unlikely to vary in different geographical locations. Conversely,

the effectiveness depends on both manufacturing factors (choice of venoms, immunological titre) and circumstantial factors (quality and quantity of inoculated venom, patient's physical condition, delay of treatment, etc.). However, following initial preclinical testing, both effectiveness and dose-finding studies may need to be repeated for a new geographical location, depending upon the similarity of the snake species in the new location with those where the antivenom was initially tested. If the species are similar, if preclinical testing indicates good neutralization, and if evidence of clinical effectiveness has been reported in other places, post-marketing surveillance studies may be adequate.

20.2.1 Dose-finding studies

Dose-finding studies seek to establish the optimum initial dose of an antivenom required to control envenoming in patients with different severities of envenoming. The therapeutic dose of an antivenom administered by the intravenous route depends on:

- the quantity of venom injected (assessed by clinical and laboratory outcomes);
- the neutralizing potency of the antivenom (given by preclinical tests); and
- the dose regimen.

The dose is calculated to neutralize a certain amount of venom and does not vary between adults and children. Preclinical testing may be used to estimate starting doses and these dosage regimens may be evaluated in a number of ways using standard effectiveness and safety end-points. Dose regimens can be assessed approximately by using prospective observational studies (*105*). High-quality observational studies may extend evidence over a wider population and are particularly useful in defining safety or when RCTs are unethical or impracticable. In these, the proportion of patients with good clinical outcomes (for example, restoration of blood coagulability or failure to develop local wound necrosis) can be observed with different, escalating or de-escalating doses of antivenom. However, the many weaknesses of observational studies must always be borne in mind.

As part of the design of the study, it is important to determine the minimum number of patients required to establish meaningful results by using sample size calculations (*168*). Results may sometimes be compared to those of previous studies (historical controls) to determine how the effectiveness or safety of a newly introduced antivenom compares with previously used antivenoms (*169*). However, such comparisons are susceptible to many kinds of confounding variables and are potentially unreliable. Subsequently, the minimum dose that

appears to be effective can be evaluated in larger Phase II trials or compared to another antivenom or a different dose in Phase III RCTs.

20.2.2 Randomized controlled trials

Definitive Phase III RCTs may require large numbers of patients because of considerable individual variation in the clinical manifestation of envenoming (or the great variability in the quantity and quality of venom injected in different patients). The new antivenom is compared with the existing standard antivenom treatment or, if none exists, two different doses of the test antivenom may be compared. Placebo controls are rarely justified unless there is genuine uncertainty about the risk and benefits of antivenom treatment. In this situation, as a safeguard against unnecessary morbidity in either treatment group, a restricted sequential plan might be incorporated (*170*) which allows evaluation of results as the trial progresses, as in the early trials of therapeutic tetanus antitoxin (*171*).

To avoid bias, patients should be randomly allocated to the groups and the study should be blinded, at a minimum to those research personnel who are assessing the clinical response and ideally to both investigators and participants. The number of patients required in each trial arm should be calculated to give the study sufficient statistical power. These power calculations are based on the expected difference in outcome between the treatment groups if the study is designed to demonstrate superiority of one treatment over another. Alternatively, predefined limits of the acceptable performance compared to an existing product are set if the trial is designed to demonstrate that the new antivenom is not worse than existing products (non-inferiority). All patients enrolled in an RCT and randomly allocated to treatment should be included in the analysis of results according to the principle of "intention to treat", so that any deleterious effects of an antivenom are not concealed by the recipients dropping out of the trial.

20.2.3 Effectiveness end-points for antivenom trials

The assessment criteria (end-points) used for antivenom studies should be predefined a priori and objective. They may be clinical or assessed by laboratory investigations. Common end-points include mortality, development of local tissue effects of envenoming such as necrosis, time taken to restore blood coagulability (assessed by the 20 minute whole blood clotting test) (*172*), other laboratory parameters such as the prothrombin time, halting of bleeding or objective clinical improvement in neurotoxicity. Surrogate markers such as platelet count are less suitable as they may be affected by complement activation resulting from the antivenom treatment itself. Patients should be observed carefully for long enough to reveal evidence of recurrent envenoming (seen particularly with short half-life Fab antivenoms) (*173*).

However, owing to the high variability of the mode of action of venoms and of the individual patient's responses, as well as the diagnostic capacity of health centres, particularly in developing countries, it is necessary to promote clinical research to identify appropriate clinical and laboratory criteria.

20.2.4 Safety end-points for antivenom trials

Because antivenoms consist of foreign proteins/fragments that are liable to aggregation, adverse effects are an inevitable risk in therapy. Appropriate manufacturing steps can reduce the rate of adverse reactions. Rates of reaction are correlated with the purity of the antivenom product and the amount of protein infused. Continuous clinical observation at the bedside is necessary for several hours after treatment to detect acute reactions; late adverse reactions may occur several weeks later. Accurate reaction rates can only be assessed prospectively. Reaction rates may differ considerably between different antivenoms, but in most cases only a small proportion of reactions are life-threatening. Although there is no consensus on classifying or grading early adverse reactions, studies should aim to detect both early adverse events (anaphylaxis and pyrogenicity) occurring at the time of, or within 24 hours of, antivenom administration (such as urticaria itching, fever, hypotension or bronchospasm) and late reactions such as serum sickness occurring between 5 and 24 days after antivenom administration (for example, fever, urticaria, arthralgia, lymphadenopathy, proteinuria or neuropathy).

20.2.5 Challenges in clinical testing of antivenoms

Several particular features of snake-bite make clinical testing of antivenoms challenging. These features include the large variation in the consequences of envenoming between individuals that make it necessary to study large numbers of patients, difficulties in identification of the species responsible for envenoming and the inaccessibility and logistical challenges of areas where snake-bite is sufficiently common to provide sufficient numbers of patients to study. Clinical studies may also be expensive, particularly multicentre studies, with the attendant additional complexity and logistics of between-centre variations. However, despite these difficulties, a number of RCTs have been undertaken and published since 1974 (*89*, *93*, *104*, *172*, *174–178*).

20.3 Post-marketing surveillance

Phase IV studies may be of much greater importance for antivenoms than for other products. A period of active post-licensing surveillance should follow:

- the introduction of a new antivenom (often a regulatory requirement);

- the introduction of changes in manufacturing processes or in the use of raw materials (for example, switching from the use of venoms produced in wild-caught snakes to venoms from captive specimens in a serpentarium), which may result in changes in the quality or effectiveness of an established antivenom;[15]
- the introduction of an established antivenom into a new geographical area.

Post-marketing studies of antivenoms examine effectiveness as well as the frequency of immediate or delayed side-effects. The combination of preclinical testing and post-marketing surveillance studies is a minimum acceptable clinical evaluation when an existing antivenom is used in a new region.

20.3.1 Possible approaches

Passive surveillance is currently practised by some antivenom manufacturers. However, approaches that rely upon voluntary return of questionnaires about safety and effectiveness are unlikely to provide the high quality data that are necessary. There are three potential approaches to obtaining such data as outlined below.

20.3.1.1 National or regional system for post-marketing surveillance

Countries using antivenoms should establish a national or regional system for the post-marketing surveillance of antivenoms. Clinicians and health workers (such as those working in poison centres) should be encouraged to report actively to national control authorities and manufacturers any unexpected lack of clinical effectiveness and any adverse reactions. These should include both early adverse events, occurring at the time of, or within 24 hours of, antivenom administration, and late reactions occurring between 5 and 24 days later. The mechanism for reporting (such as the use of standardized forms), the receiving body (for example, the national control authority), the deadline for reporting and the type of adverse events that are reportable need to be clearly defined by the authority and will depend on its structure and resources. The manufacturer of the antivenom and the authorities should assess these reports and, in consultation with one another and with specialists in the field, attempt to evaluate their significance. This assessment may require the testing of products already released and the

[15] Major changes in the design, manufacturing process, or source of venoms used for production of antivenom may necessitate new preclinical and clinical trials of a product. Such changes may also have licensing implications depending on the legislated regulations in the country of manufacture, or the countries where the product will be marketed and used.

inspection of production and control facilities and local distribution channels. If an imported product is associated with adverse reactions, the manufacturer and the national control authorities both in the country of distribution and the country of origin should be notified.

20.3.1.2 Observational studies

In certain situations, for example, the first use of an established antivenom in a new geographical area, or when routine surveillance has identified safety or effectiveness concerns, there is a rationale for setting up observational studies to ensure adequate effectiveness and safety. In the case of first use of an established antivenom in a new geographical area, such studies should follow preclinical testing that ensures neutralization of locally important venoms. Observational studies should carefully document the clinical responses to antivenom, the clinical outcomes and the frequency of reactions in a substantial cohort of patients (*179*). However, owing to inherent weaknesses of these non-randomized trials, results of observational studies may be misleading.

20.3.1.3 Sentinel sites

In some settings, where post-marketing surveillance of the whole of a country may be problematic, the use of sentinel sites may allow focusing of limited resources to maximize surveillance effectiveness.

20.3.2 Responses to results of post-marketing studies

High-quality post-marketing studies will allow clinicians, public health officials and manufacturers to identify antivenoms with poor effectiveness (batch variations in potency and safety), instances of incorrect use and dosage of antivenoms, and serious safety issues arising from the use of antivenoms. In some situations, these issues may be addressed by improving training of staff in the management of snake-bite, but these studies may also allow identification of the use of an inappropriate antivenom (*180*).

20.4 Main recommendations

- **Preclinical and clinical testing of antivenoms has been largely neglected in the past. Despite challenges, clinical trials of antivenoms in human patients have proved feasible and useful. As far as possible, trials should adhere to the principles of WHO and ICH GCP and should measure robust, objective end-points.**
- **NRAs should expect producers either to provide data confirming the clinical effectiveness and safety of their antivenoms against**

envenoming by local species of venomous snakes or, to support in-country clinical testing of these products.

- **Incorporating robust methodologies for reliable identification of the biting snake species is absolutely essential to the design of all clinical trials and other clinical studies of antivenoms.**
- **Prospective observational studies are of some use in monitoring the effectiveness and safety of an antivenom when first used in a new geographical region.**
- **Post-marketing surveillance studies should play a major role in the evaluation of effectiveness and safety of antivenoms.**

21. Role of national regulatory authorities

NRAs or medicines regulatory authorities play a crucial role in ensuring that pharmaceuticals, vaccines, biological and other medicinal products that are available for use in a country have been carefully and thoroughly evaluated against internationally recognized standards of safety and quality. These agencies of government are vital to the process of strengthening health systems by providing regulatory controls based on legislative frameworks and technical expertise. NRAs therefore have a pivotal role in ensuring the quality, safety and efficacy of antivenoms.

WHO Guidelines for national authorities on quality assurance for biological products and on regulation and licensing of biological products in countries with newly developing regulatory authorities (*181*, *182*) state that NRAs should ensure that available biological products, whether imported or manufactured locally, are of good quality, safe and efficacious, and should thus ensure that manufacturers adhere to approved standards regarding quality assurance and GMP. The responsibilities should also include the enforcement and implementation of effective national regulations, and the setting of appropriate standards and control measures. The evaluation and control of the quality, safety and consistency of production of animal-derived blood products involve the evaluation of the starting material, production processes and test methods to characterize batches of the product.

This requires the regulatory authorities to have appropriate expertise. WHO provides Member States with support in the establishment of NRAs and with the development of regulatory functions, technical abilities and adoption of standards and best practice guidelines, such as this document. A model protocol for the production and testing of snake antivenom immunoglobulins to assist NRAs in reviewing the quality of antivenom batches is provided in Appendix 2.

21.1 Regulatory evaluation of antivenoms

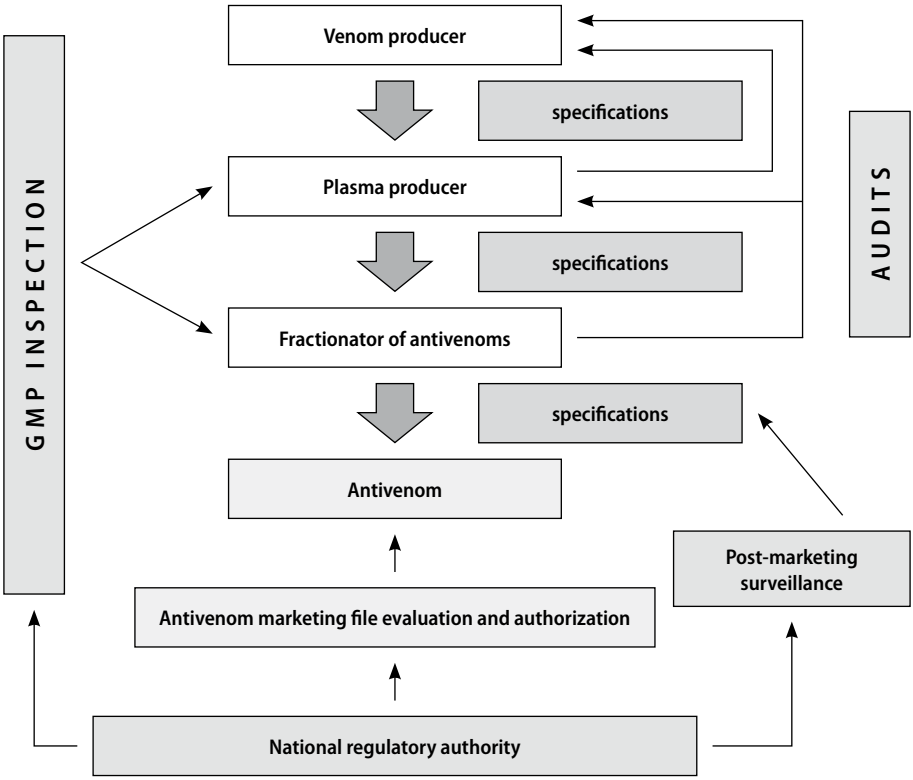
The regulatory evaluation and control of the quality, safety and consistency of production of antivenoms is summarized in Fig. A5.10 and involves the evaluation and approval of:

- the preparation of the starting plasma material from immunized animals (including the preparation of snake venom batches representative of the venomous animals of the geographical region the antivenom is made for), and the animal husbandry, control and traceability of the immunized animals and of the immunization process;
- the fractionation process used to produce the antivenoms;
- the test methods used to control batches of the product including realistic and validated potency tests based on neutralization of likely maximal envenomation;
- shelf-life and stability testing of intermediates and final product;
- the preclinical data supporting the expected effectiveness of the products for treatment of local envenomings;
- the clinical effectiveness of locally manufactured or imported antivenoms against the species of snakes found in the country, through active marketing surveillance.

21.2 Establishment licensing and site inspections

Many NRAs implement control systems based on licensing manufacturing establishments, inspecting them regularly, and enforcing the implementation of the legal requirements and applicable standards. This applies to the preparation of snake venoms, production of animal hyperimmune plasma for fractionation, and the manufacturing process of the antivenoms. Establishments involved in any or all stages of the manufacture of antivenoms need to have an establishment licence and to be inspected by the competent NRA before operations commence. To obtain the licence, the establishments have to fulfil a defined set of requirements to guarantee that their operation ensures the safety, quality and clinical effectiveness of the antivenoms.

Fig. A5.10
Schematic diagram for the regulatory evaluation and control of the quality, safety and standards of production of antivenoms



21.3 Impact of good manufacturing practices

Implementing the principles of GMP in the production of therapeutic products is acknowledged as essential for assuring the quality and safety of biological medicinal products. For antivenoms, GMP becomes even more important and more complex due to the biological nature of the production process and the complexity and local specificities of snake envenoming.

Therefore, taking into account the principles of GMP, and the existence of an appropriate quality assurance system to address and implement these requirements at all stages of manufacture, should be pivotal in ensuring the quality and safety of antivenoms. The following benefits are expected:

- ensures the application of quality assurance principles at all steps involved in the preparation of snake venoms, the production of animal plasma and the fractionation process of antivenoms;

- reduces errors and technical problems at all stages of manufacture of plasma for fractionation and antivenoms;
- contributes to the release of products that comply with quality and safety requirements;
- ensures adequate documentation and full traceability of plasma for fractionation and antivenom production stages;
- enables continuous improvement in production of plasma for fractionation and antivenoms;
- provides suitable tools for NRAs to assess the compliance status of a manufacturer of antivenoms, either local or abroad;
- supports regional cooperation networks that may result in the formation of competence centres by centralizing activities to enable compliance to be achieved at the required level.

An establishment licensing system for antivenom manufacturers operated by the responsible and competent NRA should therefore exist. The main requirements to be met to obtain an establishment licence may include in particular:

- quality assurance system and GMP applied to all steps of venom and antivenom production;
- personnel directly involved in collection, testing, processing, storage and distribution of antivenoms are appropriately qualified and provided with timely and relevant training;
- adequate premises and equipment are available;
- an adequate control system to ensure traceability of antivenoms manufacture is to be enforced through accurate identification procedures, record maintenance, and an appropriate labelling system;
- the existence of a post-marketing information system.

21.4 Inspections and audit systems in the production of antivenoms

The ongoing operations of antivenom manufacturers need to be subject to regulatory authority control and supervision in accordance with legislation. Regulatory authorities can also make use of WHO technical support services, monographs and guidelines to assist them in developing the capacity to undertake ongoing inspection activities, audits and reviews of manufacturing, quality control and other production process systems.

All manufacturers must have in place a quality assurance system for manufacture of animal-derived plasma products that comprehensively covers

all stages leading to the finished product, from production of plasma (including venom sourcing and preparation, production animal sourcing, selection, immunization and animal health control) to the collection and fractionation of the plasma into the finished products and their control. Manufacturers of antivenoms must maintain complete Site Master Files containing specific, factual details of the application of GMP to production and quality control activities that are undertaken at every site of operations linked to the products they produce. Manufacturers should also maintain quality manuals that define and describe the quality system, the scope and operations of the quality system at all levels of production, management responsibilities, key quality systems processes and safeguards. For individual products a product dossier in CTD format as recommended by WHO and ICH may also be required. NRAs should make full use of these three forms of production documentation in preparing for and conducting site inspections and audits.

For local producers, the NRA should enforce the implementation of GMP with the aim of ensuring the compliance of the manufacturer with the existing provisions. It is the responsibility of the NRA inspector to ensure that manufacturers adhere to the approved standards of GMP and quality assurance. The inspection and enforcement of control measures for venom producers, immune plasma producers, fractionation facilities and final product producers and distributors should be carried out by officials representing the competent NRA. They should be familiar with biological product technologies and trained in GMP inspections.

Inspections should follow common inspection procedures. These include:

- an opening meeting with management and key personnel;
- a tour of the facility, including inspection of the main areas and activities, such as:
  - (a) serpentariums;
  - (b) animal husbandry practices;
  - (c) animal identification and suitability for blood or plasma collection;
  - (d) process of collection and storage of blood or plasma;
  - (e) plasma fractionation process;
  - (f) testing and availability of test results for venoms, antivenoms and raw materials;
  - (g) storage, transportation and shipment; and
  - (h) quality assurance (including internal audits and change control);

- documentation (SOPs, records, donor record files, log books); personnel and organization;
- qualification and process validations;
- error and corrective action systems, recalls and complaints and product quality controls;
- a final meeting summarizing the inspection outcome.

A thorough inspection includes the observation of staff during performance of operations and comparison with established SOPs. The inspection should not only be considered as checking compliance with GMP, but also as an indirect product quality assessment by checking product-specific validation and quality control data.

A written report should summarize the main findings of the inspection including its scope, a description of the company, the deficiencies listed, specified and classified (for example, as critical, major or minor), and a conclusion. The written report is sent to the manufacturer. The manufacturers are requested to notify the NRA of the specific steps being taken, or which are planned, to correct the failures and to prevent their recurrence. If necessary, follow-up inspections should be performed – for example, to check the successful implementation of specific corrective actions. The NRA should have the mandate to withdraw an establishment licence in a case where inspection results show critical noncompliance with the requirements or product specifications. In the procedure for granting marketing authorization for an antivenom, information on the collection and control of the venoms and of the starting animal blood or plasma needs to be documented as part of the dossier. In summary, the enforcement and implementation of licensing and inspection regulatory systems for antivenoms constitute fundamental tools to ensure the quality of antivenoms produced or distributed to treat envenomings in a country.

21.5 Antivenom licensing

All antivenoms that are in use in a country must be approved and licensed by the appropriate NRA or another competent authority with legal jurisdiction. The process for applying for, considering and making a decision on the merits of an application should follow established processes and be subject to transparency and review. The process of product dossier assessment and review should be defined by legislation and appropriate regulations. Marketing authorization (licensing) of antivenoms should be subject to a thorough review of the product dossier, Site Master File and quality management system. For a product that is to be imported, NRAs should communicate directly with the licensing authority in the country of manufacturer to ensure that claims in the documents are factual and the product meets licensing requirements in its country of origin.

21.6 National reference venoms

As discussed in section 10.2 countries or regions should establish collections of reference venoms ("standard venoms") against which antivenom products and the venoms used by manufacturers can be assessed. The establishment of reference venoms for release control of final product should be reviewed and monitored by the regulatory authority or by other competent authorities with technical expertise in the production of international reference material standards. Antivenom manufacturers should not be involved in the production of reference standards in order to ensure transparency. The task should be assigned to a central quality control laboratory, or to a third-party organization with specific expertise. The potency of each batch of final product should be confirmed by specific neutralization of a standard venom of each species of snake for which the antivenom is indicated.

A system of control for the reference venoms and for the design of the venom pools (for example, the geographical selection of animals) should be in place as part of the procedures for the management of reference venom collections.

21.7 Main recommendations

- **NRAs should regulate and supervise local antivenom manufacturers.**
- **NRAs are responsible for market authorization of antivenoms distributed in the country.**
- **Inspection and audit processes are fundamental to the effective regulation and control of antivenoms and NRAs should seek appropriate assistance to develop both the legislative and technical expertise necessary to undertake these functions.**
- **Only antivenoms that pass stringent applications processes should be granted market authorization.**
- **National reference collections of "standard venoms" should be established according to accepted international reference material standards, and used to independently assess antivenoms, or to validate venoms used by manufacturers.**

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Prior to their presentation to the sixty-seventh meeting of the WHO Expert Committee on Biological Standardization, held in Geneva, Switzerland, from 17 to 21 October 2016, the draft Guidelines were published on the WHO Biologicals website as part of a process of public consultation. The draft document was also emailed to: all second edition section reviewers; antivenom manufacturers; regulatory authorities in countries producing antivenoms; WHO regional offices (for further distribution in each region); and all members of the Committee, with a request to provide feedback and comments.

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Further changes were subsequently made to document WHO/BS/2016.2300 by the WHO Expert Committee on Biological Standardization.

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Appendix 1

Worldwide distribution of medically important venomous snakes

Venomous snakes are widely distributed, especially in tropical countries, from sea level to altitudes of up to 4900 metres (*Gloydius himalayanus*). The European adder (*Vipera berus*) enters the Arctic Circle, and the Argentine Yararanata (*Bothrops ammodytoides*) occurs to 47 °S and is the most southerly occurring venomous snake. No other venomous species occur in cold regions such as the Arctic, Antarctic and north of around latitude 51 °N in North America (Newfoundland, Nova Scotia).

This Appendix lists venomous snake species considered to represent the greatest threat to public health in various countries, territories and other areas or regions around the world. Only species which fall into one of the two categories listed below are shown, and category listings are in alphabetical order according to taxonomic family, genus and species. The intention in categorizing these medically important snakes into two groups is to provide users of these WHO Guidelines with a prioritized listing. Snakes in both Category 1 and Category 2 are species for which antivenom production is important; however species listed in Category 1 within a country, territory or area should be considered as being of highest priority for antivenom production on the basis that available knowledge implicates them as being responsible for the greater burden in that particular setting.

Definitions of the categories used in this listing are:

- **CATEGORY 1 (CAT 1)**: Highest medical importance

  Definition: highly venomous snakes which are common or widespread and cause numerous snake-bites, resulting in high levels of morbidity, disability or mortality.

- **CATEGORY 2 (CAT 2)**: Secondary medical importance

  Definition: highly venomous snakes capable of causing morbidity, disability or death, but: (a) for which exact epidemiological or clinical data may be lacking; and/or (b) are less frequently implicated (owing to their activity cycles, behaviour, habitat preferences or occurrence in areas remote from large human populations).

There are numerous other venomous species that rank as lesser threats in countries territories and other areas listed here, and interested readers should

refer to the herpetological references provided in these Guidelines. It should be noted that over time, as more information becomes available, new species will doubtlessly be added to these lists, and some species, currently defined within Category 1 or Category 2 will be re-ranked.

It should also be noted that the organization of countries, territories and other areas in this Appendix does not follow the WHO regional organization, but instead is arranged biogeographically in alphabetical order of country, territory or geographical area. This approach was necessary to reflect the geographical distribution of major groups of venomous snakes throughout the world. For example, the venomous snakes of the eastern Indonesian Province of Papua have biogeographical origins in Australo-Papua, and are evolutionarily distinct from the venomous snakes of Asian origin that occur west of Wallace's Line, which runs south of the Philippines, between Borneo and Sulawesi, and between Bali and Lombok, and which separates the zoogeographical regions of Asia and Australia. For this reason, the medically important snakes of Indonesian Papua are listed in the Australo-Papuan region, rather than the South-East Asian region.

Users of this Appendix should also recognize that the relative risk of injury from a particular species may vary from one country, territory or area to another. For this reason, some species that have been listed under Category 1 in one country, territory or area may have been listed under Category 2 in another country, territory or area to reflect the different risk posed by that species in different locations. Assignment to Category 1 or Category 2 was based in some cases on the relative importance of a species as a cause of snake-bite. In Europe, for example, the overall incidence of snake-bite is trivial compared to that in West Africa or India, but where a European species (such as *Vipera berus*) is a major (or sole) cause of envenoming where it occurs, this warrants ranking it as a medically important species in that setting.

AFRICA AND THE MIDDLE EAST

Island populations

Off the coast of Africa, there are no medically important snakes in Mauritius, Réunion, Rodrigues, the Comoros, the Canary Islands, the Cabo Verde Islands or the Seychelles. The islands that do have venomous snakes include the Lamu group, Zanzibar, Pemba and Mafia Islands, the Bazaruto Archipelago and Inhaca Island, São Tomé, Principe, Bioko (Fernando Po) and Dahlak Islands. The venomous snakes on these islands tend to be similar to those on the adjacent mainland. A colubrid, *Madagascarophis meridionalis*, and perhaps other species of the same genus, are the only terrestrial snakes of possible, if minimal, medical importance found in Madagascar.

North Africa/Middle East

Algeria:

Cat 1:	Elapidae: ***Naja haje***; Viperidae: ***Cerastes cerastes***; ***Daboia mauritanica***
Cat 2:	Viperidae: *Daboia deserti*; *Echis leucogaster*; *Macrovipera lebetina*; *Vipera latastei*

Cyprus:

Cat 1:	**None**
Cat 2:	Viperidae: *Macrovipera lebetina*

Egypt:

Cat 1:	Elapidae: ***Naja haje***; Viperidae: ***Cerastes cerastes***; ***Echis coloratus*** (east), ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis engaddensis* (Sinai); Elapidae: *Naja nubiae*; *Walterinnesia aegyptia* (Sinai); Viperidae: *Pseudocerastes fieldi*

Iraq:

Cat 1:	Viperidae: ***Echis carinatus***; ***Macrovipera lebetina***
Cat 2:	Elapidae: *Walterinnesia morgani*; Viperidae: *Cerastes gasperettii*; *Pseudocerastes fieldi*, *Pseudocerastes persicus*

Iran (Islamic Republic of):

Cat 1:	Elapidae: ***Naja oxiana***; Viperidae: ***Echis carinatus***; ***Macrovipera lebetina***; ***Pseudocerastes persicus***
Cat 2:	Elapidae: *Bungarus persicus* (south-east); *Walterinnesia morgani* (west); Viperidae: *Eristicophis macmahonii* (east); *Gloydius halys caucasicus*; *Montivipera raddei*; *Vipera spp.*

Israel:

Cat 1:	Viperidae: ***Daboia palaestinae***; ***Echis coloratus***
Cat 2:	Atractaspididae: *Atractaspis engaddensis*; Elapidae: *Walterinnesia aegyptia*; Viperidae: *Cerastes cerastes*, *Cerastes gasperettii*; *Pseudocerastes fieldi*

Jordan:

Cat 1:	Viperidae: ***Daboia palaestinae***; ***Echis coloratus***
Cat 2:	Atractaspididae: *Atractaspis engaddensis*; Elapidae: *Walterinnesia aegyptia*; Viperidae: *Cerastes gasperettii*; *Macrovipera lebetina*; *Pseudocerastes fieldi*

Kuwait and Qatar:

Cat 1:	Viperidae: ***Cerastes gasperettii***
Cat 2:	Elapidae: *Walterinnesia morgani* (Kuwait only)

Lebanon:

Cat 1:	Viperidae: ***Daboia palaestinae***; ***Macrovipera lebetina***
Cat 2:	None

Libya:

Cat 1:	Elapidae: ***Naja haje***; Viperidae: ***Cerastes cerastes***; ***Echis pyramidum***
Cat 2:	Viperidae: *Daboia deserti*

Morocco:

Cat 1:	Elapidae: ***Naja haje***; Viperidae: ***Bitis arietans***; ***Cerastes cerastes***; ***Daboia mauritanica***
Cat 2:	Viperidae: *Echis leucogaster*; *Vipera latastei*

Oman:

Cat 1:	Atractaspididae: ***Atractaspis andersonii*** (south-west); Viperidae: ***Bitis arietans*** (south-west); ***Echis coloratus*** (south-west), ***Echis carinatus***, ***Echis omanensis*** (north)
Cat 2:	Elapidae: *Naja arabica* (south-west); Viperidae: *Cerastes gasperettii*; *Echis khosatzkii* (south-west); *Pseudocerastes persicus*

Saudi Arabia:

Cat 1:	Atractaspididae: ***Atractaspis andersonii*** (south-west); Viperidae: ***Cerastes gasperettii***; ***Echis coloratus***, ***Echis borkini*** (south-west)
Cat 2:	Atractaspididae: *Atractaspis engaddensis* (north-west); Elapidae: *Naja arabica* (south-west); *Walterinnesia aegyptia* (west), *Walterinnesia morgani* (central and south); Viperidae: *Bitis arietans* (south-west); *Cerastes cerastes* (south-west); *Pseudocerastes fieldi*

Syrian Arab Republic:

Cat 1:	Viperidae: ***Daboia palaestinae***; ***Macrovipera lebetina***
Cat 2:	Viperidae: *Pseudocerastes fieldi*

Tunisia:

Cat 1:	Viperidae: ***Daboia mauritanica***
Cat 2:	Elapidae: *Naja haje*; Viperidae: *Cerastes cerastes*; *Daboia deserti*; *Echis leucogaster*; *Macrovipera lebetina*; *Vipera latastei*

Turkey:

Cat 1:	Viperidae: ***Macrovipera lebetina***; ***Montivipera xanthina***
Cat 2:	Elapidae: *Walterinnesia morgani* (south); Viperidae: *Montivipera raddei*; *Vipera ammodytes*, *Vipera eriwanensis*, *Vipera spp.*

United Arab Emirates:

Cat 1:	Viperidae: ***Echis carinatus*** (east); ***Echis omanensis***
Cat 2:	Viperidae: *Cerastes gasperettii*; *Pseudocerastes persicus*

West Bank and Gaza Strip:

Cat 1:	Viperidae: ***Daboia palaestinae***; ***Echis coloratus***
Cat 2:	Atractaspididae: *Atractaspis engaddensis*; Elapidae: *Walterinnesia aegyptia*; Viperidae: *Cerastes cerastes*; *Pseudocerastes fieldi*

Western Sahara:

Cat 1:	Viperidae: ***Cerastes cerastes***
Cat 2:	Elapidae: *Naja haje*; Viperidae: *Bitis arietans*

Yemen:

Cat 1:	Atractaspididae: ***Atractaspis andersonii***; Elapidae: ***Naja arabica***; Viperidae: ***Bitis arietans***; ***Echis borkini***, ***Echis coloratus***
Cat 2:	Viperidae: *Cerastes cerastes*, *Cerastes gasperettii*; *Echis khosatzkii*

Central sub-Saharan Africa

Angola:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***, ***Dendroaspis polylepis***; ***Naja anchietae***, ***Naja melanoleuca***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***
Cat 2:	Atractaspididae: *Atractaspis bibronii*, *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis kirtlandii* (north); Elapidae: *Naja christyi* (Cabinda), *Naja mossambica* (south), *Naja nigricincta* (south-west); *Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis nasicornis* (Cabinda)

Burundi:

Cat 1:	Elapidae: ***Naja nigricollis***, ***Naja melanoleuca***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*, *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis mossambicanus*; Elapidae: *Dendroaspis jamesoni*; Viperidae: *Bitis gabonica*, *Bitis nasicornis*

Central African Republic:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***, ***Dendroaspis polylepis***; ***Naja haje***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***; ***Echis ocellatus***, ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Naja annulata*, *Naja melanoleuca*;[1] *Pseudohaje goldii*; Viperidae: *Atheris broadleyi*, *Atheris squamigera*; *Bitis nasicornis*

Chad:

Cat 1:	Elapidae: ***Naja haje***, ***Naja nigricollis***; Viperidae: ***Bitis arietans*** (south); ***Echis ocellatus*** (south)
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Naja katiensis*, *Naja nubiae*; Viperidae: *Cerastes cerastes*

Republic of the Congo:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja melanoleuca***; Viperidae: ***Bitis gabonica***, ***Bitis nasicornis***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Naja annulata*, *Naja christyi*, *Naja nigricollis*; *Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis arietans*

Democratic Republic of the Congo:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja melanoleuca***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***, ***Bitis nasicornis***
Cat 2:	Atractaspididae: *Atractaspis bibronii*, *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis kirtlandii*; Elapidae: *Dendroaspis polylepis*; *Naja anchietae* (Katanga pedicle), *Naja annulata*, *Naja christyi*, *Naja haje* (north); *Pseudohaje goldii*; Viperidae: *Atheris squamigera*

[1] The medical importance of this species may be higher in the primary forest zone of the south-western Central African Republic, and in some secondary forest mosaic zones elsewhere in the Central African Republic.

Equatorial Guinea:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja melanoleuca***; Viperidae: ***Bitis gabonica***, ***Bitis nasicornis***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Thelotornis kirtlandii*; Elapidae: *Naja annulata*; *Pseudohaje goldii*; Viperidae: *Atheris squamigera*

Gabon:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja melanoleuca***, ***Naja nigricollis***; Viperidae: ***Bitis gabonica***, ***Bitis nasicornis***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Thelotornis kirtlandii*; Elapidae: *Naja annulata*; *Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis arietans*

Rwanda:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja nigricollis***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*, *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Dendroaspis polylepis*; *Naja annulata*, *Naja melanoleuca*; *Pseudohaje goldii*; Viperidae: *Bitis gabonica*, *Bitis nasicornis*

East sub-Saharan Africa

Djibouti:

Cat 1:	Viperidae: ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis fallax*; Colubridae: *Dispholidus typus*; Elapidae: *Naja pallida*; Viperidae: *Bitis arietans*

Eritrea:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja haje***; Viperidae: ***Bitis arietans***; ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Elapidae: *Naja nubiae*; Viperidae: *Echis megalocephalus*

Ethiopia:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja ashei*** (south-east), ***Naja haje***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis fallax*, *Atractaspis irregularis* (Mt Bizen); Colubridae: *Dispholidus typus*; Elapidae: *Naja melanoleuca*, *Naja pallida*; Viperidae: *Bitis parviocula*, *Bitis harenna*

Kenya:

Cat 1:	Elapidae: ***Dendroaspis angusticeps***, ***Dendroaspis polylepis***; ***Naja ashei*** (north & east), ***Naja haje***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis bibronii*, *Atractaspis fallax*, *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis mossambicanus*, *Thelotornis usambaricus* (east coast); Elapidae: *Dendroaspis jamesoni*; *Naja melanoleuca* (west and coastal forest), *Naja pallida* (north and east); *Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis nasicornis*, *Bitis gabonica* (west)

Malawi:

Cat 1:	Elapidae: ***Dendroaspis angusticeps***, ***Dendroaspis polylepis***; ***Naja annulifera***, ***Naja mossambica***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis mossambicanus*; Elapidae: *Naja melanoleuca*; Viperidae: *Proatheris superciliaris*

Mozambique:

Cat 1:	Elapidae: ***Dendroaspis angusticeps***, ***Dendroaspis polylepis***; ***Naja annulifera***, ***Naja mossambica***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis mossambicanus*; Elapidae: *Hemachatus haemachatus*; *Naja melanoleuca*; Viperidae: *Proatheris superciliaris*

Somalia:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja ashei*** (south), ***Naja haje***; Viperidae: ***Bitis arietans***; ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis fallax*; Colubridae: *Dispholidus typus*; *Thelotornis mossambicanus*; Elapidae: *Naja pallida*, *Naja melanoleuca*; Viperidae: *Echis hughesi* (north)

South Sudan:

Cat 1:	Elapidae: ***Naja haje***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis pyramidum***
Cat 2:	Atractaspididae: *Atractaspis fallax*, *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis jamesoni*, *Dendroaspis polylepis*; *Naja melanoleuca*, *Naja nubiae*, *Naja pallida*; Viperidae: *Bitis gabonica*, *Bitis nasicornis*

Sudan:

Cat 1:	Elapidae: ***Naja haje***; Viperidae: ***Bitis arietans***; ***Echis pyramidum***
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis polylepis* (east); *Naja nubiae*; Viperidae: *Cerastes cerastes*, *Echis coloratus* (east)

United Republic of Tanzania:

Cat 1:	Elapidae: ***Dendroaspis angusticeps***, ***Dendroaspis polylepis***; ***Naja mossambica*** (including Pemba Island), ***Naja nigricollis***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*, *Atractaspis fallax* (north), *Atractaspis irregularis* (north-east); Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis kirtlandii* (Mahali and Udzungwa Mountains), *Thelotornis mossambicanus*, *Thelotornis usambaricus* (East Usambara Mountains); Elapidae: *Naja ashei* (poss. north-east), *Naja annulata*, *Naja haje* (north), *Naja melanoleuca* (west and coast, including Mafia Island), *Naja pallida*; Viperidae: *Atheris squamigera*; *Bitis gabonica* (west and south-east), *Bitis nasicornis* (north); *Proatheris superciliaris*

Uganda:

Cat 1:	Elapidae: ***Naja ashei*** (north-east), ***Naja haje*** (north), ***Naja nigricollis***; ***Dendroaspis jamesoni***, ***Dendroaspis polylepis***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Naja melanoleuca*; *Pseudohaje goldii*; Viperidae: *Atheris squamigera*; *Bitis nasicornis*

Zambia:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja anchietae***, ***Naja annulifera***, ***Naja mossambica***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis kirtlandii*, *Thelotornis mossambicanus*; Elapidae: *Naja annulata*, *Naja melanoleuca*

South sub-Saharan Africa

Botswana:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja anchietae*** (west), ***Naja annulifera*** (east), ***Naja mossambica***, ***Naja nivea*** (south-west); Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*

Lesotho:

Cat 1:	Elapidae: ***Naja nivea***; Viperidae: ***Bitis arietans***
Cat 2:	Elapidae: *Hemachatus haemachatus*

Namibia:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja anchietae***, ***Naja nivea*** (central and southern), ***Naja mossambica*** (north-east), ***Naja nigricincta***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*; Elapidae: *Naja nigricollis* (Caprivi)

South Africa:

Cat 1:	Elapidae: ***Dendroaspis angusticeps*** (Natal), ***Dendroaspis polylepis***; ***Naja annulifera*** (north-east), ***Naja nivea***, ***Naja mossambica*** (north-east); Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*; Elapidae: *Hemachatus haemachatus*; *Naja melanoleuca* (KwaZulu-Natal), *Naja nigricincta* (north-west); Viperidae: *Bitis gabonica* (KwaZulu-Natal)

Swaziland:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja annulifera***, ***Naja mossambica***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*; Elapidae: *Hemachatus haemachatus*

Zimbabwe:

Cat 1:	Elapidae: ***Dendroaspis polylepis***; ***Naja anchietae*** (west), ***Naja annulifera***, ***Naja mossambica***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis bibronii*; Colubridae: *Dispholidus typus*; *Thelotornis capensis*, *Thelotornis mossambicanus*; Elapidae: *Dendroaspis angusticeps* (east); *Hemachatus haemachatus* (Nyanga Mountains); *Naja melanoleuca* (east); Viperidae: *Bitis gabonica* (east)

West sub-Saharan Africa

Benin:

Cat 1:	Elapidae: ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis ocellatus***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis jamesoni*; *Naja katiensis*, *Naja melanoleuca*, *Naja senegalensis*; *Pseudohaje nigra*; Viperidae: *Bitis rhinoceros*; *Echis leucogaster* (far north)

Burkina Faso:

Cat 1:	Elapidae: ***Naja nigricollis***, ***Naja katiensis***; Viperidae: ***Bitis arietans***; ***Echis ocellatus***
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis polylepis*; *Naja melanoleuca*, *Naja senegalensis*; Viperidae: *Echis leucogaster*

Cameroon:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja haje***, ***Naja nigricollis***, ***Naja melanoleuca***;[2] Viperidae: ***Bitis arietans***, ***Bitis gabonica***, ***Bitis nasicornis***; ***Echis ocellatus***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Dendroaspis polylepis*; *Naja annulata*, *Naja katiensis*; *Pseudohaje goldii*; Viperidae: *Atheris broadleyi* (East Province), *Atheris squamigera*

Côte d'Ivoire:

Cat 1:	Elapidae: ***Dendroaspis viridis***; ***Naja nigricollis***, ***Naja melanoleuca***, ***Naja senegalensis***; Viperidae: ***Bitis arietans***, ***Bitis nasicornis***, ***Bitis rhinoceros***; ***Echis ocellatus***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Dendroaspis polylepis*; *Naja katiensis*; *Pseudohaje goldii*, *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*

Gambia:

Cat 1:	Elapidae: ***Dendroaspis viridis***; ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis jogeri***
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Naja katiensis*, *Naja melanoleuca*, *Naja senegalensis*

[2] This large, highly venomous snake is common in forested areas of south-west Cameroon and a high burden of injury may be expected, although clinical data with direct attribution are not yet available.

Ghana:

Cat 1:	Elapidae: ***Dendroaspis viridis***; ***Naja nigricollis***, ***Naja senegalensis***; Viperidae: ***Bitis arietans***; ***Echis ocellatus***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*, *Thelotornis kirtlandii*; Elapidae: *Naja katiensis*, *Naja melanoleuca*;[3] *Pseudohaje goldii*, *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*; *Bitis nasicornis*, *Bitis rhinoceros*

Guinea:

Cat 1:	Elapidae: ***Dendroaspis polylepis***, ***Dendroaspis viridis***; ***Naja katiensis***, ***Naja nigricollis***, ***Naja melanoleuca***, ***Naja senegalensis***; Viperidae: ***Bitis arietans***; ***Echis jogeri***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*; *Bitis nasicornis*, *Bitis rhinoceros*

Guinea-Bissau:

Cat 1:	Elapidae: ***Dendroaspis viridis***; ***Naja nigricollis***, ***Naja melanoleuca***, ***Naja senegalensis***; Viperidae: ***Bitis arietans***; ***Echis jogeri***
Cat 2:	Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Viperidae: *Bitis rhinoceros*

Liberia:

Cat 1:	Elapidae: ***Dendroaspis viridis***; ***Naja melanoleuca***, ***Naja nigricollis***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Thelotornis kirtlandii*; Elapidae: *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*; *Bitis nasicornis*, *Bitis rhinoceros*

Mali:

Cat 1:	Elapidae: ***Naja katiensis***, ***Naja nigricollis***, ***Naja senegalensis***; Viperidae: ***Bitis arietans***; ***Echis jogeri*** (west), ***Echis leucogaster***, ***Echis ocellatus***
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Naja melanoleuca*; Viperidae: *Cerastes cerastes*

Mauritania:

Cat 1:	Elapidae: ***Naja senegalensis*** (south-east); Viperidae: ***Cerastes cerastes***; ***Echis leucogaster***
Cat 2:	Viperidae: *Bitis arietans*

[3] The medical importance of this species may be higher in the forested zone of southern Ghana.

Niger:

Cat 1:	Elapidae: ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis leucogaster***, ***Echis ocellatus***
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Naja haje* (south-central), *Naja katiensis*, *Naja nubiae*; *Naja senegelensis* (south-west); Viperidae: *Cerastes cerastes*

Nigeria:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja haje*** (north-east), ***Naja nigricollis***; Viperidae: ***Bitis arietans***, ***Bitis gabonica***; ***Echis ocellatus***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Naja katiensis*, *Naja melanoleuca*,[4] *Naja senegalensis* (north-west); *Pseudohaje goldii*, *Pseudohaje nigra*; Viperidae: *Atheris squamigera*; *Bitis nasicornis*; *Echis leucogaster* (north)

Sao Tome and Principe:

Cat 1:	Elapidae: ***Dendroaspis jamesoni***; ***Naja melanoleuca***
Cat 2:	None

Senegal:

Cat 1:	Elapidae: ***Naja katiensis***, ***Naja nigricollis***; Viperidae: ***Bitis arietans***; ***Echis leucogaster***, ***Echis jogeri***
Cat 2:	Colubridae: *Dispholidus typus*; Elapidae: *Dendroaspis polylepis*, *Dendroaspis viridis*; *Naja melanoleuca*, *Naja senegalensis*

Sierra Leone:

Cat 1:	Elapidae: ***Dendroaspis viridis***; ***Naja nigricollis***; Viperidae: ***Bitis arietans***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Naja melanoleuca*;[5] *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*; *Bitis nasicornis*, *Bitis rhinoceros*

[4] The medical importance of this species may be higher in the southern rainforest belt of Nigeria, from Ibadan in the west to Oban and Eket in the east, and in the forested southern quarter of Sierra Leone.

[5] The medical importance of this species may be higher in the forested southern quarter of Sierra Leone.

Togo:

Cat 1:	Elapidae: ***Naja nigricollis***, ***Naja senegalensis***; Viperidae: ***Bitis arietans*** (south); ***Echis ocellatus***
Cat 2:	Atractaspididae: *Atractaspis irregularis*; Colubridae: *Dispholidus typus*; *Thelotornis kirtlandii*; Elapidae: *Dendroaspis jamesoni*, *Dendroaspis viridis*; *Naja katiensis*, *Naja melanoleuca*; *Pseudohaje goldii*, *Pseudohaje nigra*; Viperidae: *Atheris chlorechis*; *Bitis nasicornis*, *Bitis rhinoceros*

ASIA AND AUSTRALASIA

Central Asia

Armenia:

Cat 1:	Viperidae: ***Macrovipera lebetina***
Cat 2:	Viperidae: *Montivipera raddei*; *Vipera eriwanensis*, *Vipera* spp.

Azerbaijan:

Cat 1:	Viperidae: ***Macrovipera lebetina***
Cat 2:	Viperidae: *Gloydius halys*; *Vipera eriwanensis*, *Vipera* spp.

Georgia:

Cat 1:	Viperidae: ***Macrovipera lebetina***; ***Vipera ammodytes***
Cat 2:	Viperidae: *Vipera kaznakovi*, *Vipera renardi*, *Vipera* spp.

Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan and Turkmenistan:

Cat 1:	Elapidae: ***Naja oxiana*** (except Kazakhstan and Kyrgyzstan); Viperidae: ***Echis carinatus*** (except Kyrgyzstan); ***Macrovipera lebetina*** (except Kazakhstan and Kyrgyzstan); ***Gloydius halys*** (throughout)
Cat 2:	Viperidae: *Vipera renardi* (except Turkmenistan)

Mongolia:

Cat 1:	Viperidae: *Gloydius halys*
Cat 2:	Viperidae: *Vipera berus*, *Vipera renardi*

East Asia

China:

China mainland

Cat 1:	Elapidae: ***Bungarus multicinctus***; ***Naja atra***; Viperidae: ***Trimeresurus albolabris***; ***Daboia siamensis***; ***Deinagkistrodon acutus***; ***Gloydius brevicaudus***; ***Protobothrops mucrosquamatus***
Cat 2:	Colubridae: *Rhabdophis tigrinus*; Elapidae: *Bungarus bungaroides* (south-east Tibet), *Bungarus fasciatus*; *Naja kaouthia*; *Ophiophagus hannah*; Viperidae: *Trimeresurus septentrionalis* (south Tibet); *Gloydius halys*, *Gloydius intermedius*, *Gloydius ussuriensis*; *Himalayophis tibetanus* (south Tibet); *Protobothrops jerdonii*, *Protobothrops kaulbacki*, *Protobothrops mangshanensis*; *Vipera berus* (Jilin, western Xinjiang), *Vipera renardi* (western Xinjiang); *Trimeresurus stejnegeri*

Hong Kong, Special Administrative Region

Cat 1:	Elapidae: ***Bungarus multicinctus***; ***Naja atra***; Viperidae: ***Trimeresurus albolabris***
Cat 2:	None

Taiwan Province

Cat 1:	Elapidae: ***Bungarus multicinctus***; ***Naja atra***; Viperidae: ***Protobothrops mucrosquamatus***; ***Trimeresurus stejnegeri***
Cat 2:	Viperidae: *Deinagkistrodon acutus*; *Daboia siamensis*

Democratic People's Republic of Korea:

Cat 1:	Viperidae: ***Gloydius brevicaudus***
Cat 2:	Viperidae: *Gloydius intermedius*, *Gloydius ussuriensis*; *Vipera berus*

Japan (including Ryukyu Islands):

Cat 1:	Viperidae: ***Gloydius blomhoffii*** (main islands); ***Protobothrops flavoviridis*** (Ryukyu Islands)
Cat 2:	Colubridae: *Rhabdophis tigrinus*; Viperidae: *Gloydius tsushimaensis* (Tsushima); *Protobothrops elegans*

Republic of Korea:

Cat 1:	Viperidae: ***Gloydius brevicaudus***
Cat 2:	Colubridae: *Rhabdophis tigrinus*; Viperidae: *Gloydius intermedius*, *Gloydius ussuriensis*

South Asia

Afghanistan:

Cat 1:	Elapidae: ***Naja oxiana***; Viperidae: ***Echis carinatus***; ***Macrovipera lebetina***
Cat 2:	Elapidae: *Bungarus caeruleus* (east), *Bungarus sindanus* (east); *Naja naja* (poss. south-east); Viperidae: *Eristicophis macmahonii* (south-west); *Gloydius halys* (north)

Bangladesh:

Cat 1:	Elapidae: ***Bungarus caeruleus***, ***Bungarus niger***, ***Bungarus walli***; ***Naja kaouthia***; Viperidae: ***Trimeresurus erythrurus***
Cat 2:	Elapidae: *Bungarus bungaroides*, *Bungarus fasciatus*, *Bungarus lividus*; *Naja naja*; *Ophiophagus hannah*; Viperidae: *Trimeresurus albolabris* (far north-west); *Daboia russelii* (west)

Bhutan:

Cat 1:	Elapidae: ***Bungarus niger***; ***Naja naja***
Cat 2:	Elapidae: *Bungarus caeruleus*, *Bungarus fasciatus*, *Bungarus lividus*; *Naja kaouthia*; *Ophiophagus hannah*; Viperidae: *Trimeresurus erythrurus*; *Daboia russelii*; *Protobothrops jerdonii*

India:

Cat 1:	Elapidae: ***Bungarus caeruleus***; ***Naja kaouthia*** (east), ***Naja naja*** (throughout); Viperidae: ***Daboia russelii***; ***Echis carinatus***; ***Hypnale hypnale*** (south-west)
Cat 2:	*Bungarus bungaroides*, *Bungarus fasciatus*, *Bungarus lividus*, *Bungarus niger*, *Bungarus sindanus*, *Bungarus walli*; *Naja oxiana* (west), *Naja sagittifera* (Andaman Islands); *Ophiophagus hannah* (south, north-east, Andaman Islands); Viperidae: *Trimeresurus albolabris*, *Trimeresurus erythrurus*, *Trimeresurus septentrionalis*; *Gloydius himalayanus*; *Protobothrops jerdonii*, *Protobothrops kaulbacki*, *Protobothrops mucrosquamatus*; *Trimeresurus gramineus* (south India), *Trimeresurus malabaricus* (south-west),

Nepal:

Cat 1:	Elapidae: ***Bungarus caeruleus***, ***Bungarus niger***; ***Naja naja***, ***Naja kaouthia***; Viperidae: ***Daboia russelii***
Cat 2:	Elapidae: *Bungarus bungaroides*, *Bungarus fasciatus*, *Bungarus lividus*, *Bungarus walli*; *Ophiophagus hannah*; Viperidae: *Trimeresurus septentrionalis*; *Gloydius himalayanus*; *Himalayophis tibetanus*; *Protobothrops jerdonii*

Pakistan:

Cat 1:	Elapidae: ***Bungarus caeruleus***, ***Bungarus sindanus***; ***Naja naja***, ***Naja oxiana***; Viperidae: ***Daboia russelii***; ***Echis carinatus***
Cat 2:	Viperidae: *Eristicophis macmahonii* (west); *Gloydius himalayanus* (north); *Macrovipera lebetina* (west)

Sri Lanka:

Cat 1:	Elapidae: ***Bungarus caeruleus***; ***Naja naja***; Viperidae: ***Daboia russelii***; ***Hypnale hypnale***
Cat 2:	Elapidae: *Bungarus ceylonicus*; Viperidae: *Echis carinatus*; *Hypnale nepa*, *Hypnale zara*; *Trimeresurus trigonocephalus*

South-East Asia

Brunei Darussalam:

Cat 1:	Elapidae: ***Naja sumatrana***
Cat 2:	Elapidae: *Bungarus fasciatus*, *Bungarus flaviceps*; *Calliophis bivirgatus*, *Calliophis intestinalis*; *Ophiophagus hannah*; Viperidae: *Trimeresurus sumatranus*; *Tropidolaemus subannulatus*

Cambodia:

Cat 1:	Elapidae: ***Bungarus candidus***; ***Naja kaouthia***, ***Naja siamensis***; Viperidae: ***Calloselasma rhodostoma***; ***Trimeresurus albolabris***; ***Daboia siamensis***
Cat 2:	Elapidae: *Bungarus fasciatus*, *Bungarus flaviceps*; *Ophiophagus hannah*; Viperidae: *Trimeresurus cardamomensis*

Indonesia (Sumatra, Java, Borneo, Sulawesi and Lesser Sunda Islands):

Cat 1:	Elapidae: ***Bungarus candidus*** (Sumatra and Java); ***Naja sputatrix*** (Java and Lesser Sunda Islands), ***Naja sumatrana*** (Sumatra and Borneo); Viperidae: ***Calloselasma rhodostoma*** (Java); ***Trimeresurus albolabris***; ***Daboia siamensis***
Cat 2:	Elapidae: *Bungarus fasciatus*, *Bungarus flaviceps* (Sumatra and Borneo); *Calliophis bivirgatus*, *Calliophis intestinalis*; *Ophiophagus hannah* (Sumatra, Borneo & Java); Viperidae: *Trimeresurus insularis*, *Trimeresurus purpureomaculatus* (Sumatra), *Trimeresurus sumatranus*; *Tropidolaemus subannulatus*

Lao People's Democratic Republic:

Cat 1:	Elapidae: ***Bungarus candidus***, ***Bungarus multicinctus***; ***Naja atra*** (north), ***Naja siamensis*** (south and east); Viperidae: ***Calloselasma rhodostoma***; ***Trimeresurus albolabris***
Cat 2:	Elapidae: *Bungarus fasciatus*; *Naja kaouthia* (south and east); *Ophiophagus hannah*; Viperidae: *Trimeresurus macrops*; *Protobothrops jerdonii*, *Protobothrops mucrosquamatus*

Malaysia:

Cat 1:	Elapidae: ***Bungarus candidus*** (Peninsular Malaysia); ***Naja kaouthia*** (northern Peninsular Malaysia), ***Naja sumatrana*** (Peninsular Malaysia, Sabah and Sarawak); Viperidae: ***Calloselasma rhodostoma***
Cat 2:	Elapidae: *Bungarus fasciatus*, *Bungarus flaviceps*; *Calliophis bivirgatus*, *Calliophis intestinalis*; *Ophiophagus hannah*; Viperidae: *Trimeresurus purpureomaculatus*, *Trimeresurus hageni*; *Tropidolaemus subannulatus*

Myanmar:

Cat 1:	Elapidae: ***Bungarus magnimaculatus***, ***Bungarus multicinctus***; ***Naja kaouthia***, ***Naja mandalayensis***; Viperidae: ***Trimeresurus albolabris***, ***Trimeresurus erythrurus***; ***Daboia siamensis***
Cat 2:	Elapidae: *Bungarus bungaroides* (Chin State), *Bungarus candidus* (Thaninthayi Div.), *Bungarus flaviceps* (east Shan State), *Bungarus niger*; *Ophiophagus hannah*; Viperidae: *Calloselasma rhodostoma* (Thaninthayi Div.); *Trimeresurus purpureomaculatus*; *Protobothrops jerdonii*, *Protobothrops kaulbacki*, Protobothrops mucrosquamatus (Kachin)

Philippines:

Cat 1:	Elapidae: ***Naja philippinensis*** (Luzon), ***Naja samarensis*** (Mindanao), ***Naja sumatrana*** (Palawan)
Cat 2:	Elapidae: *Calliophis intestinalis*; *Ophiophagus hannah*; Viperidae: *Trimeresurus flavomaculatus*; *Tropidolaemus philippensis*, *Tropidolaemus subannulatus*

Singapore:

Cat 1:	Elapidae: ***Bungarus candidus***; ***Naja sumatrana***
Cat 2:	Elapidae: *Bungarus fasciatus*; *Calliophis bivirgatus*, *Calliophis intestinalis*; *Ophiopahgus hannah*; Viperidae: *Trimeresurus purpureomaculatus*

Thailand:

Cat 1:	Elapidae: ***Bungarus candidus***; ***Naja kaouthia***, ***Naja siamensis***; Viperidae: ***Calloselasma rhodostoma***; ***Trimeresurus albolabris***; ***Daboia siamensis***
Cat 2:	Elapidae: *Bungarus fasciatus*, *Bungarus flaviceps*; *Calliophis bivirgatus*, *Calliophis intestinalis*; *Naja sumatrana*; *Ophiophagus hannah*; Viperidae: *Trimeresurus macrops*, *Trimeresurus hageni*

Timor-Leste:

Cat 1:	Viperidae: ***Trimeresurus insularis***
Cat 2:	None

Viet Nam:

Cat 1:	Elapidae: ***Bungarus candidus***, ***Bungarus multicinctus***, ***Bungarus slowinskii*** (north); ***Naja atra*** (north), ***Naja kaouthia*** (south); Viperidae: ***Calloselasma rhodostoma***; ***Trimeresurus albolabris*** (throughout)
Cat 2:	Elapidae: *Bungarus fasciatus*, *Bungarus flaviceps* (south); *Naja siamensis* (south); *Ophiophagus hannah*; Viperidae: *Trimeresurus rubeus*; *Protobothrops jerdonii*, *Protobothrops mucrosquamatus* (north); *Trimeresurus stejnegeri*; *Deinagkistrodon acutus*

Australo-Papua *(including Pacific Islands)*:

There are no medically important land snakes in American Samoa, Cook Islands, Fiji, French Polynesia, Guam, Kiribati, Marshall Islands, Nauru, New Caledonia, New Zealand, Northern Mariana Islands, Pitcairn Island, Samoa, Tokelau, Tonga, Tuvalu, or Wallis and Futuna Islands. Fiji possesses a single terrestrial venomous snake species (*Ogmodon vitianus*) while the Solomon Islands possess three terrestrial venomous species (*Salomonelaps par*; *Loveridgelaps elapoides* and *Parapistocalamus hedigeri*) associated with no and few snake-bites, respectively.

Australia:

Cat 1:	Elapidae: ***Notechis scutatus***; ***Pseudechis australis***;[6] ***Pseudonaja affinis***, ***Pseudonaja mengdeni***, ***Pseudonaja nuchalis***, ***Pseudonaja textilis***
Cat 2:	Elapidae: *Acanthophis antarcticus*, *Acanthophis cryptamydros*, *Acanthophis* spp.; *Austrelaps spp.*; *Hoplocephalus* spp.; *Oxyuranus microlepidotus*, *Oxyuranus scutellatus*, *Oxyuranus temporalis*; *Pseudechis* spp.; *Pseudonaja aspidorhyncha*, *Pseudonaja* spp.; *Tropidechis carinatus*

[6] *Pseudechis australis* is common and widespread and causes numerous snake-bites; bites may be severe, although this species has not caused a fatality in Australia since 1968.

Indonesia (West Papua and Maluku):

Cat 1:	Elapidae: ***Acanthophis laevis***
Cat 2:	Elapidae: *Acanthophis rugosus*; *Micropechis ikaheka*; *Oxyuranus scutellatus*; *Pseudechis papuanus*, *Pseudechis rossignolii*; *Pseudonaja textilis*

Papua New Guinea:

Cat 1:	Elapidae: ***Acanthophis laevis***; ***Oxyuranus scutellatus***
Cat 2:	Elapidae: *Acanthophis rugosus*; *Micropechis ikaheka*; *Pseudonaja textilis*; *Pseudechis papuanus*, *Pseudechis rossignolii*

EUROPE

There are no venomous snakes in Iceland, Ireland, Isle of Man, Outer Hebrides, Orkney or the Shetland Islands. Crete and most of the islands of the western Mediterranean are also free of venomous snakes.

Central Europe

Albania, Bulgaria, Romania, Serbia, Montenegro, Slovenia, The former Yugoslav Republic of Macedonia:

Cat 1:	Viperidae: ***Vipera ammodytes***
Cat 2:	Viperidae: *Vipera berus*

Bosnia and Herzegovina:

Cat 1:	Viperidae: ***Vipera ammodytes***
Cat 2:	Viperidae: *Vipera berus*

Croatia:

Cat 1:	Viperidae: ***Vipera ammodytes***
Cat 2:	Viperidae: *Vipera berus*

Czechia:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Greece:

Cat 1:	Viperidae: ***Vipera ammodytes***
Cat 2:	Viperidae: *Macrovipera schweizeri*; *Montivipera xanthina*; *Vipera berus*

Hungary:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Poland:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Slovakia:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Eastern Europe

Belarus, Estonia, Latvia, Lithuania, Republic of Moldova:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*, *Vipera nikolskii* (Moldova)

Russian Federation:

Cat 1:	Viperidae: *Vipera berus*
Cat 2:	Viperidae: *Gloydius halys*, *Gloydius intermedius*, *Gloydius ussuriensis* (far-east Russia); *Macrovipera lebetina* (Dagestan); *Vipera nikolskii*, *Vipera renardi*, *Vipera* spp.

Ukraine:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*, *Vipera nikolskii*, *Vipera renardi*

Western Europe

Austria:

Cat 1:	None
Cat 2:	Viperidae: *Vipera ammodytes*, *Vipera berus*

Belgium:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Denmark:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Finland:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

France:

Cat 1:	Viperidae: ***Vipera aspis***
Cat 2:	Viperidae: *Vipera berus*

Germany:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Italy:

Cat 1:	Viperidae: ***Vipera aspis***
Cat 2:	Viperidae: *Vipera ammodytes*, *Vipera berus*

the Netherlands:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Norway:

Cat 1:	None
Cat 2:	Viperidae: *Vipera berus*

Portugal:

Cat 1:	None
Cat 2:	Viperidae: *Vipera latastei*, *Vipera seoanei*

Spain:

Cat 1:	None
Cat 2:	Viperidae: *Vipera aspis*, *Vipera latastei*, *Vipera seoanei*

Sweden:

Cat 1:	Viperidae: ***Vipera berus***
Cat 2:	None

Switzerland:

Cat 1:	None
Cat 2:	Viperidae: *Vipera aspis*, *Vipera berus*

United Kingdom of Great Britain and Northern Ireland:

Cat 1:	Viperidae: ***Vipera berus*** (not Northern Ireland)
Cat 2:	None

THE AMERICAS

North America

Canada:

Cat 1:	None
Cat 2:	Viperidae: *Crotalus oreganus*, *Crotalus viridis*; *Sistrurus catenatus*

Mexico:

Cat 1:	Viperidae: ***Agkistrodon bilineatus***, ***Agkistrodon taylori***; ***Crotalus atrox***, ***Crotalus scutulatus***, ***Crotalus simus***, ***Crotalus molossus***; ***Bothrops asper***
Cat 2:	Elapidae: *Micruroides euryxanthus*, *Micrurus nigrocinctus*, *Micrurus tener*, *Micrurus* spp.; Viperidae: *Agkistrodon contortrix*, *Agkistrodon russeolus*; *Atropoides mexicanus*, *Atropoides occiduus*, *Atropoides* spp.; *Bothriechis schlegelii*, *Bothriechis* spp.; *Cerrophidion godmani*, *Cerrophidion* spp.; *Crotalus basiliscus*, *Crotalus totonacus*, *Crotalus oreganus*, *Crotalus ruber*, *Crotalus tzabcan*, *Crotalus viridis*, *Crotalus* spp.; *Ophryacus* spp.; *Porthidium nasutum*, *Porthidium* spp.; *Sistrurus catenatus*

United States of America:

Cat 1:	Viperidae: ***Agkistrodon contortrix***, ***Agkistrodon piscivorus***; ***Crotalus adamanteus***, ***Crotalus atrox***, ***Crotalus horridus***, ***Crotalus oreganus***, ***Crotalus scutulatus***, ***Crotalus viridis***
Cat 2:	Elapidae: *Micrurus fulvius*, *Micrurus tener*; Viperidae: *Crotalus molossus*, *Crotalus ornatus*, *Crotalus ruber*, *Crotalus* spp.; *Sistrurus catenatus*, *Sistrurus miliarius*

Central America

The most medically important species are Crotalus simus and Bothrops asper.

Belize:

Cat 1:	Viperidae: ***Bothrops asper***
Cat 2:	Elapidae: *Micrurus* spp.; Viperidae: *Agkistrodon russeolus*; *Atropoides mexicanus*; *Bothriechis schlegelii*; *Crotalus tzabcan*; *Porthidium nasutum*

Costa Rica:

Cat 1:	Viperidae: ***Bothrops asper***; ***Crotalus simus***
Cat 2:	Elapidae: *Micrurus nigrocinctus*, *Micrurus* spp.; Viperidae: *Agkistrodon howardgloydi*; *Atropoides mexicanus*, *Atropoides picadoi*; *Bothriechis schlegelii*, *Bothriechis lateralis*, *Bothriechis* spp.; *Cerrophidion sasai*; *Lachesis melanocephala*, *Lachesis stenophrys*; *Porthidium nasutum*, *Porthidium ophrymegas*, *Porthidium* spp.

El Salvador:

Cat 1:	Viperidae: ***Crotalus simus***
Cat 2:	Elapidae: *Micrurus nigrocinctus*; *Micrurus* spp.; Viperidae: *Agkistrodon bilineatus*; *Atropoides occiduus*; *Bothriechis* spp.; *Cerrophidion wilsoni*; *Porthidium ophryomegas*

Guatemala:

Cat 1:	Viperidae: ***Bothrops asper***; ***Crotalus simus***
Cat 2:	Elapidae: *Micrurus nigrocinctus*, *Micrurus* spp.; Viperidae: *Agkistrodon bilineatus*, *Agkistrodon russeolus*; *Atropoides mexicanus*, *Atropoides occiduus*; *Bothriechis schlegelii*, *Bothriechis* spp.; *Cerrophidion godmani*; *Crotalus tzabcan*, *Porthidium nasutum*, *Porthidium ophryomegas*

Honduras:

Cat 1:	Viperidae: ***Bothrops asper***
Cat 2:	Elapidae: *Micrurus nigrocinctus*, *Micrurus* spp.; Viperidae: *Agkistrodon howardgloydi*; *Atropoides mexicanus*, *Atropoides* spp.; *Bothriechis marchi*, *Bothriechis schlegelii*, *Bothriechis* spp.; *Cerrophidion wilsoni*; *Crotalus simus*; *Porthidium nasutum*, *Porthidium ophryomegas*

Nicaragua:

Cat 1:	Viperidae: ***Bothrops asper***; ***Crotalus simus***
Cat 2:	Elapidae: *Micrurus nigrocinctus*, *Micrurus* spp.; Viperidae: *Agkistrodon howardgloydi*; *Atropoides mexicanus*; *Bothriechis schlegelii*; *Cerrophidion godmani*; *Lachesis stenophrys*; *Porthidium nasutum*, *Porthidium ophryomegas*

Panama:

Cat 1:	Viperidae: ***Bothrops asper***
Cat 2:	Elapidae: *Micrurus mipartitus*, *Micrurus nigrocinctus*, *Micrurus* spp.; Viperidae: *Atropoides mexicanus*, *Atropoides* spp.; *Bothriechis lateralis*, *Bothriechis schlegelii*, *Bothriechis* spp.; *Cerrophidion sasai*; *Lachesis acrochorda*, *Lachesis stenophrys*; *Porthidium nasutum*, *Porthidium lansbergii*, *Porthidium* spp.

Caribbean

No medically important snakes occur naturally in Anguilla, Antigua and Barbuda, the Bahamas, Barbados, Bermuda, The British Virgin Islands, Cayman Islands, Cuba, Dominica, the Dominican Republic, Grenada, Guadeloupe, Haiti, Jamaica, Montserrat, the Netherlands Antilles, Saint Kitts and Nevis, Saint Vincent and the Grenadines, and Turks and Caicos Islands.

Aruba, Martinique, Saint Lucia, Trinidad and Tobago, and offshore islands:

Cat 1:	Viperidae: ***Bothrops* cf. *atrox*** (Trinidad), ***Bothrops caribbaeus*** (St Lucia), ***Bothrops lanceolatus*** (Martinique); ***Crotalus durissus*** (Aruba)
Cat 2:	Elapidae: *Micrurus circinalis* (Trinidad), *Micrurus lemniscatus* (Trinidad); Viperidae: *Lachesis muta* (Trinidad)

South America

No venomous snakes are occur naturally in the Falkland Islands and no dangerously venomous snakes occur naturally in Chile.

Argentina:

Cat 1:	Viperidae: ***Bothrops alternatus***, ***Bothrops diporus***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus corallinus*, *Micrurus lemniscatus*, *Micrurus* spp.; Viperidae: *Bothrops ammodytoides*, *Bothrops jararaca*, *Bothrops jararacussu*, *Bothrops mattogrossensis*, *Bothrops neuwiedi*, *Bothrops pubescens*

Bolivia (Plurinational State of):

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops mattogrossensis***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus spixii*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothrocophias hyoprora*, *Bothrocophias microphthalmus*; *Bothrops bilineatus*, *Bothrops brazili*, *Bothrops jararacussu*, *Bothrops jonathani*, *Bothrops moojeni*, *Bothrops sanctaecrucis*, *Bothrops* spp., *Bothrops taeniatus*; *Lachesis muta*

Brazil:

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops jararaca***, ***Bothrops jararacussu***, ***Bothrops leucurus***, ***Bothrops moojeni***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus corallinus*, *Micrurus lemniscatus*, *Micrurus spixii*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothrocophias hyoprora*; *Bothrocophias microphthalmus*; *Bothrops alternatus*, *Bothrops bilineatus*, *Bothrops brazili*, *Bothrops diporus*, *Bothrops mattogrossensis*, *Bothrops neuwiedi*, *Bothrops pubescens*, *Bothrops taeniatus*, *Bothrops* spp.; *Lachesis muta*

Colombia:

Cat 1:	Viperidae: ***Bothrops asper***, ***Bothrops atrox***, ***Bothrops bilineatus***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus mipartitus*, *Micrurus nigrocinctus*, *Micrurus spixii*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothriechis schlegelii*; *Bothrocophias hyoprora*, *Bothrocophias microphthalmus*, *Bothrocophias* spp.; *Bothrops brazili*, *Bothrops taeniatus*, *Bothrops* spp.; *Lachesis acrochorda*, *Lachesis muta*; *Porthidium nasutum*, *Porthidium lansbergii*

Ecuador:

Cat 1:	Viperidae: ***Bothrops asper***, ***Bothrops atrox***, ***Bothrops bilineatus***; ***Lachesis muta***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus mipartitus*, *Micrurus spixii*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothriechis schlegelii*; *Bothrocophias hyoprora*, *Bothrocophias microphthalmus*, *Bothrocophias* spp.; *Bothrops brazili*, *Bothrops taeniatus*, *Bothrops* spp.; *Lachesis acrochorda*; *Porthidium nasutum*, *Porthidium* spp.

French Guiana (France):

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops bilineatus***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothrops brazili*, *Bothrops taeniatus*; *Lachesis muta*

Guyana:

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops bilineatus***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothrops brazili*, *Bothrops taeniatus*; *Lachesis muta*

Paraguay:

Cat 1:	Viperidae: ***Bothrops alternatus***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus corallinus*, *Micrurus lemniscatus*, *Micrurus spixii*, *Micrurus* spp.; Viperidae: *Bothrops diporus*, *Bothrops jararaca*, *Bothrops jararacussu*, *Bothrops mattogrossensis*, *Bothrops moojeni*, *Bothrops neuwiedi*, *Bothrops* spp.

Peru:

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops bilineatus***, ***Bothrops pictus***; ***Crotalus durissus***; ***Lachesis muta***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus mipartitus*, *Micrurus spixii*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothriechis schlegelii*; *Bothrocophias hyoprora*, *Bothrocophias microphthalmus*; *Bothrops asper*; *Bothrops brazili*, *Bothrops mattogrossensis*, *Bothrops taeniatus*, *Bothrops* spp.

Suriname:

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops bilineatus***; ***Crotalus durissus***
Cat 2:	Elapidae: *Micrurus lemniscatus*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothrops brazili*, *Bothrops taeniatus*; *Lachesis muta*

Uruguay:

Cat 1:	Viperidae: ***Bothrops alternatus***; ***Bothrops pubescens***
Cat 2:	Elapidae: *Micrurus corallinus*, *Micrurus* spp.; Viperidae: *Crotalus durissus*

Venezuela (Bolivarian Republic of):

Cat 1:	Viperidae: ***Bothrops atrox***, ***Bothrops* cf. *atrox***, ***Bothrops venezuelensis***; ***Crotalus durissus*** (including Isla de Margarita)
Cat 2:	Elapidae: *Micrurus circinalis*, *Micrurus lemniscatus*, *Micrurus mipartitus*, *Micrurus spixii*, *Micrurus surinamensis*, *Micrurus* spp.; Viperidae: *Bothriechis schlegelii*; *Bothrops asper*, *Bothrops brazili*, *Bothrops bilineatus*; *Lachesis muta*; *Porthidium lansbergii*

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Appendix 2

Model protocol for the production and testing of snake antivenom immunoglobulins

Identification of the lot

Name and address of manufacturer _____

_____

Lot number of antivenom _____
Date of filling _____
Liquid or freeze-dried _____
Expiry date _____
Number of vials or ampoules _____
Temperature of storage _____

Control of the venom batch(es) used for animal immunization

Producer of venom and location _____
Information on the snake contributing to the venom batch _____
Scientific names of the snake species _____
Number of snakes _____
Geographical origins of the snakes _____
Dates of collection of the venoms _____
Expiry date of the venoms preparation _____
Biochemical and biological characterization of the venoms _____
- Test performed _____
- Results _____

Control of plasma donor animals

Location of the animal herd _____
Animal species used for immunization _____
Vaccinations performed on animals _____
Dates of immunization _____
Control of antivenom antibody titre of animal _____
Veterinary certificate of health of animal donor _____

Collection and storage of plasma

Method of collection _____
Date of collection _____
Date of storage _____
Type of containers _____
Temperature of storage _____
Type and content of preservatives added (if any) _____

Transport of plasma to fractionation facility

Date of transport _____
Temperature of transport _____
Date of arrival _____

Plasma pooling and fractionation

Temperature of plasma storage at fractionation facility _____
Volume of plasmas of different specificity pooled for the production of polyspecific antivenoms (if applicable) _____
Date of plasma pooling _____
Volume of the manufacturing plasma pool _____
Number of animal donors contributing to the manufacturing plasma pool _____
Quality control of manufacturing plasma pool _____
- Test performed _____
- Results _____

Type of active substance (intact IgG, fragments) _____

Preparation and control of final bulk

Volume of bulk antivenoms of different specificity pooled for the production of polyspecific antivenoms (if applicable) _____
Concentration of preservatives (if used) _____
- Type _____
- Method _____
- Result _____

Quality control of manufacturing plasma pool _____
- Test performed _____
- Results _____

Filling and containers

Date of filling _____
Quantity of containers _____
Volume of antivenoms per container _____
Date of freeze-drying (if applicable) _____

Control tests on final product

Appearance _____
Solubility (freeze-dried product) _____
Extractable volume _____
Venom-neutralizing potency assay
- Method _____
- Venom used _____
- Results _____

Osmolality _____
Identity test
- Method _____
- Result _____

Protein concentration
- Method _____
- Result _____

Purity
- Method _____
- Result _____

Molecular size distribution
- Method _____
- Result _____

Test for pyrogenic substances
- Method _____
- Result _____

Sterility test
No. of containers examined _____
- Method _____
- Date at start of test _____
- Date at end of test _____

Concentration of sodium chloride and other excipients
- Method _____
- Result _____

Determination of pH

- Result _____

Concentration of preservatives (if used)

- Type _____
- Method _____
- Result _____

Chemical agents used in plasma fractionation

- Type _____
- Method _____
- Result _____

Inspection of final containers

- Results _____

Residual moisture in freeze-dried antivenoms

- Method _____
- Result _____

Internal certification

Certification by person taking overall responsibility for production of the antivenom
I certify that batch no. _____ of _____ snake antivenom immunoglobulin meets all national requirements and/or satisfies the 2016 WHO Guidelines for the production, control and regulation of snake antivenom immunoglobulins.[1]

Signature _____
Name (typed) _____
Date _____

[1] WHO Technical Report Series, No. 1004, Annex 5.