- a simple blood test, suitable for use even in peripheral health care centres, can define the biting species (e.g. 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.

However, most countries are inhabited by several medically important species of snakes, where there may be no distinctive clinical syndrome to direct the use of a monospecific antivenom. In these cases, the manufacture of polyspecific antivenoms should be highly recommended.

*6.2.2 Polyspecific antivenoms*

Some clinicians are uncertain about using polyspecific antivenoms because of a fear that they have an inherently lower potency than monospecific antivenoms but this is not necessarily the case.

Polyspecific antivenoms can be generated by immunizing animals with a mixture of venoms from various snake species. The resulting antivenom will then contain antibodies against venom components of various snake species. When a polyspecific antivenom is produced this way, by immunizing an animal with venoms from several taxonomically-related snakes (e.g. different vipers), the titre of neutralizing antibodies against individual venoms may in some cases be higher than in a monospecific antivenom produced by immunizing an animal with only a single venom (*31*).

Polyspecific antivenoms can also be obtained either by:

— immunizing individual animals with the venom of a single species and then mixing the various hyperimmune plasmas for fractionation; or

— mixing appropriate quantities of the relevant purified antivenoms before formulation.

When using the latter option it is important to monitor the potency for each monospecific antivenom to ensure that the potency of the mix in the final product is consistent, reproducible and in line with the product specification for each individual venom. A sufficiently high titre should be guaranteed to avoid the need for infusion of an additional antivenom dose to the patient, as this may increase the risks of adverse reactions. However, in such "combined antivenoms", neutralizing antibodies against all individual venoms will be proportionally diluted. In general, such dilution implies that a greater amount of antivenom proteins would be infused to patients, which is likely to increase the risks of adverse reactions.

In some regions, it is possible to differentiate envenomings on the basis of obvious distinct clinical effects: neurotoxicity, local tissue damage and/or haematological disturbances (haemorrhage or coagulopathy). Such situations justify the preparation of separate polyspecific antivenoms against mixtures of either neurotoxic venoms or venoms inflicting tissue damage, haemorrhage and/or coagulopathy.

Polyspecific antivenoms offer significant clinical advantages and their production should be encouraged, whenever technically possible. They can be produced using venoms from a range of species of venomous snakes of high medical relevance, broadening their usefulness and making identification of the biting species less critical, having the additional advantage of simplicity of distribution and supply.

### 6.3 Main recommendations

- **When selecting snake antivenoms national health authorities should first obtain and consider the information on the local species and their relative medical importance.**
- **The design of the venom mixture used in immunization, and the decision to prepare monospecific or polyspecific antivenoms, depend on the epidemiological and clinical information on snakebites in that particular region, country or area.**
- **Owing to the difficulty of identifying clinically the snake species responsible for envenoming, polyspecific antivenoms appropriate to the geographical region may be more practical and convenient to use than monospecific antivenoms.**
- **Polyspecific antivenom may be prepared either by mixing monospecific antivenoms or by immunizing animals with a mixture of venoms, provided that the specified neutralizing titre for each venom is achieved. The preparation of antivenoms by immunizing animals with a mixture of venoms from taxonomically related snakes may result in a higher titre antivenom.**
- **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 17).**

## 7 PREPARATION AND STORAGE OF SNAKE VENOM

Venom preparations are used both to hyper-immunize animals, as part of antivenom production, and to provide reference venom samples for routine and/or preclinical potency assessment of antivenoms. Ensuring their quality is therefore critical, and their preparation should follow the principles and recommendations mentioned below. The essential principles of quality systems should be followed in venom production including traceability, reproducibility, taxonomic accuracy, and hygiene control.

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 of an individual species (*32–34*), it is imperative that the venom of an adequate number of snakes (generally not less than 20–50 specimens) from the same geographical location should be collected together. A similar preparation can be used as a national standard of venoms for routine potency assessment of antivenoms (see section 8) and to perform preclinical testing of antivenoms (see section 17) to verify that the antivenom is suitable to treat envenomings in the region efficiently. Venom producers should follow rigorously the recommendations listed below and provide evidence of compliance on:

— geographical origin and size (and hence the approximate age) of each individual snake used for venom production;

— taxonomic details of each snake used;

— correct implementation of the Convention on International Trade in Endangered Species (CITES) documents in the case of endangered species;

— precautionary measures to avoid collection of venoms from sick snakes;

— individual identification of snakes contributing to each venom batch; and

— traceability of each venom batch.

Being able to fulfil the following is also strongly recommended:

— rapid freezing of the venom after collection;

— lyophilization of the venom for long-term storage[1]; and

— confirmation of batch-to-batch similarity of venom of the same origin.

## 7.1 Production of snake venoms for immunization

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

### *7.1.1 Quarantine of snakes*

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

On arrival, snakes should be examined by a specialized veterinary surgeon (or experienced person) for ectoparasites and pentastomids – which should be eliminated using broad-spectrum antiparasitic drugs – and possible infections, particularly transmissible ones (*35–37*). Some viruses can be transmitted between different species (for example from *Bothrops* spp. to *Crotalus* spp.).

Sick snakes should be treated and their quarantine extended for 2 months after complete clinical recovery. Sick animals found in "production rooms" may be treated *in situ* but they cannot be milked for venom production. If an antibiotic treatment is given, the snake should not be milked for 4 weeks following the end of the treatment. When housed in good conditions, adult snakes collected from the wild can live on a snake farm 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 (*38*).

### *7.1.2 Maintenance of captive snakes for venom production*

Individual snakes should preferably be housed in separate cages large enough to allow them to move about. 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. The selection of cleaning and disinfecting agents should be carefully considered to ensure they do not have adverse effects on the snakes. Cages should be adequately ventilated but perforations or mesh should be small enough to prevent escape. In the case of gravid female vipers, the mesh should be sufficiently fine to prevent escape of their tiny, live-born babies. 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. A disposable floor covering (e.g. 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 a single small 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

[1] Desiccation may be acceptable if proven to ensure stability of the preparation.

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 plastic or wooden, but should be readily cleanable. The roof of the hide box should be removable, to allow easy, safe extraction of the snake, when required.

Cages should be thoroughly cleaned and disinfected, ideally when soiled (perhaps almost daily for elapids), but at least every week. Faeces and uneaten or regurgitated rodents should be removed. To avoid misidentification of the snake, 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 provided on at least two days per week, but 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 milking. This room should be kept as clean as possible and thoroughly cleaned at least weekly. Access should be guarded by a tray containing an antiseptic which is placed on the floor at the entrance so that the footwear of all people entering is automatically treated. 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. They 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 to check whether any animals have escaped from their cages. The spaces below the doors should be less than 3 mm and all openings to the exterior (e.g. water pipes, drainage conduits, ventilation entrances and exits) should be protected by grilles having holes smaller than 5 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 have similar living requirements (i.e. temperature and humidity).

When kept under favourable housing and climatic conditions, and if left undisturbed, snakes will reproduce in captivity (*39*). Animals should be mated only with specimens from the same species, subspecies and local origin (*40*, *41*). 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 newborns from ovoviviparous snakes should be removed from their mother's cage as soon as possible. When a difference in the venom composition of adult and juvenile snakes has been reported, as in the case of *Bothrops* and *Crotalus* species (*32*, *42–44*), the venom of a certain proportion of juvenile snakes might be mixed with that of adults.

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 being milked, ideally with dead mice or other appropriate prey according to the snake species. Some snakes will only accept living prey, but attempts should be made to wean them onto dead prey. 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 or even snake faeces. 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 when 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 "bar code" for each snake, or, alternatively, using a reliable manual recording system, and constitute useful records related to the venom batches produced. Water should be provided in the milking room from a tap, shower or reservoir, as is the case in laboratories where there is a risk of chemical injuries.

*7.1.3 General maintenance of a snake farm*

In addition to the rooms devoted to snake housing, sufficient space should be made available for the storage of consumables, rooms for cleaning and sanitizing cages and racks, animal houses for rat and mouse production, a storage room for conservation of the venom produced, control laboratories and administrative offices.

The cage cleaning rooms should be large enough to accommodate all the cages that are being cleaned and sanitized. 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, inadvertently left in its cage, attempts to escape. 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 sacrificed in accordance with ethical principles. The rooms for rodent production should be large enough to provide sufficient numbers of rats or mice to feed the snakes. 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. If snake are bred on the farm, egg incubators, and special rooms for newborns and juveniles are required. It should be taken into account that the diets required by young snakes might differ from those of adults (for instance, frogs and tadpoles are preferred to rodents by some species).

When possible, it is useful to have a small laboratory for performing quality control on the venoms (see section 8). 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.

*7.1.4 Snake milking for venom production*

Specific safety consideration for operators should be applied to snake milking (see section 7.2). All operations should be fully described in written procedures, which should be checked and revised periodically according to a written master document. Pools of venom require unique batch numbers, and the snake milkings contributing to the pool should be traceable.

7.1.4.1 Venom collection on snake farms

Snakes can be milked according to a regular schedule, depending on the species. The interval between milkings varies among producers and ranges from every 2 or 3 weeks to every 3 months, except for specimens that are in quarantine or are undergoing treatment and snakes in the process of sloughing their skins.

Handling equipment must be appropriate for the particular species of snake to cause the least stress and must be familiar to and afford safety to the operator. The snake is gently removed from its cage with a hook and either placed on a foam rubber pad before being pinned behind the head or encouraged to crawl into a transparent plastic tube. For very dangerous species, the use of short-acting general anaesthesia, or moderate cooling (15 °C) during milking can be considered (e.g. inhaled sevofluorane or sevoflurane, halothane 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 between index finger and thumb, just behind the angle of the jaw, while the snake's body is held between the trunk and the arm of the snake handler. An assistant should gently occlude the snake's cloaca to prevent messy contamination of the locality by spraying of faeces.

By applying gentle pressure, the snake's jaws are forced open, the fangs exposed and, in the case of vipers, erected. In the case of large vipers, the dental sheath is retracted when necessary with clean forceps. The fangs are pushed through a plastic/parafilm membrane (or the snake may voluntarily strike through the membrane) hooked over the lip of a glass vessel, and venom is squeezed out. The use of siliconized containers might be considered to minimize venom attaching to the container surface. While a brief electrical impulse of moderate intensity can be applied to stimulate venom secretion, this technique is not used or required by most venom producers, although it may help in avoiding debris in the venom. Any venom sample contaminated with blood should be rejected. After venom extraction, the fangs are carefully withdrawn from the collection vessel, while preventing damage to the mouth and dentition and avoiding the snake's impaling itself with its own fangs. After each venom milking, all materials used for milking should be sterilized with a flame, then cooled with a draught of air before the next snake is milked.

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 (*45*). In the case of colubrid snakes, special techniques are required, such as application of foam rubber pads or capillary tubes to the posteriorly-placed fangs and the use of secretagogue drugs. Similarly, some elapid snakes have only small fangs capillary tubes or similar are required to collect venom. At the time of milking, there is an opportunity to remove broken or diseased fangs and to examine the snake for ectoparasites (e.g. ticks and mites), for pentastomids escaping from the snake's respiratory tract and for areas of adherent dead skin and opercular scales over the snake's eyes. The snake can be treated with drugs or vitamins at the same time and, if necessary, can be force-fed. Milking 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 milking process should be performed following clean practices.

Several snakes from the same group (same species and subspecies collected at the same time in the same area) can be milked into the same glass vessel. However, it is important for most venoms to be snap frozen at −20 °C or colder within 1 hour. One method of achieving this for venoms with high proteolytic activity, is to pour the collected venom into a vial maintained at a low temperature (ideally at −70 to −80 °C, but, if this is not possible, at −20 to −40 °C), every 10 minutes or at least every 30 minutes, before another snake from the same group is milked. Centrifugation of freshly collected venom is recommended, for instance at 1000 g for 5 minutes, since it removes cellular debris.

It is important to identify the vial into which the venom has been collected with an appropriate reference number. Primary identification must be on the vial. This allows the identification of all the snakes used, the day of the milking, the name of the operator and any other relevant information. To obtain large venom batches for the preparation of antivenom, one approach is to

use the same vial over several months for milking the same snakes, providing the cold chain is never broken. Pools of venom require unique batch numbers, and the snake milkings contributing to the pool should be traceable. The venom vial will then be freeze-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. However, some producers use an alternative system, keeping venom at 20–25 °C in a dessicator. Large pools of frozen venoms, collected from many individuals, are allowed to thaw at 0 °C, to avoid proteolytic degradation of venom components and, after being thoroughly mixed, aliquots of liquid venoms are prepared. These aliquots are then freeze-dried and stored in the dark at a low temperature (either −20 °C or 4 °C). Aliquots of freeze-dried venoms should be adequately labelled. Freeze-drying cycles should be established, followed, and documented. Venom stored for considerable periods of time should be tested to ensure that no degradation or loss of activity has occurred (see section 8).

During milking, the wearing of protective clothing and a mask as well as vinyl gloves is recommended to prevent any accidents or infections. The equipment used for storage of frozen venom (freezers) and for freeze-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 as per a defined schedule.

7.1.4.2 Venom collection from wild snakes

In some parts of the world it is accepted practice that during certain seasons, collectors from a snake farm or local snake catchers will go to designated localities in the wild and collect venom from snakes manually and release them in the same locality after milking. In such cases, collecting venoms from wild snakes may be the only alternative, provided the venom and the collection process are subject to stringent rules, including most of the procedures already described for captive animals. At any time the collectors may milk from 50 to more than a hundred snakes; usually these are snakes of smaller size e.g. *Echis* species.

If collection of venom from wild snakes is necessary, most of the steps and safety procedures which are followed in milking captive snakes should be adhered to as far as possible, with certain modifications for the field conditions. The team which goes to the field for collection should include a herpetologist or zoologist who is able to help and confirm the identity of the snakes. Sick snakes, injured snakes and gravid females should not be milked. Detailed records of the locality, season, climate, date, size and number of snakes milked in one batch or pool should be maintained. One reference specimen from this locality should be taken to the laboratory and deposited as a voucher specimen for that pool with options for traceability. Where feasible, a photographic record of snakes milked in the field should be retained.

During milking, the wearing of protective clothing and a mask as well as vinyl gloves is recommended to prevent any accidents or infections, as for venom collection on a snake farm. Snake handling and milking should be done in an environment where there is little risk of external contamination. For example, the milking could be done inside a vehicle rather than in the open field. The field team should have training in first aid as well as in administration of antivenom, or they should be within reach of a hospital with antivenom available in case accidents occur. Milked venom should be frozen as soon as possible in a freezer in the vehicle. This is particularly important for venoms having high proteolytic activity, such as many viperid venoms, to avoid enzymatic degradation of venom components.

## 7.2 Staff responsible for handling snakes

### *7.2.1 Safety and health considerations*

Handling and milking snakes is a dangerous operation. One envenoming occurred every two years in each of the 15 extraction facilities reviewed by Powell et al. (*46*). Twelve bites were recorded, 10 with envenoming, and one case of venom being squirted into the eye of a worker (*47*), between 1981 and 1999, when 370 768 venom extractions were performed at a venom production plant from *Bothrops moojeni*.

Milking should be done very carefully 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 adequately trained, and the training procedures must be documented. A minimum of two people should be present during snake handling for venom collection. For safety reasons, it is recommended that sessions for milking of snakes should be interrupted at least every 2 hours, for a rest period before re-starting the process.

### *7.2.2 Clothing for snake and venom handling*

Protective clothing should include an eye covering (plastic spectacles), especially when spitting elapids capable of squirting their venom are being handled, and a laboratory coat or gown. The wearing of protective gloves designed to prevent an effective bite is unpopular and not usually recommended because it impairs manual dexterity and sense of touch, but the use of nitrile gloves is advisable to prevent cross-contamination.

When lyophilized or desiccated venom is being handled, the safety of operators is paramount, since venom aerosols may form and affect people through skin breaks, eyes or mucous membranes, or may sensitize them to the venom. Appropriate gowning is necessary when handling lyophilized venom, to prevent contact of the venom with skin or mucous membranes.

### *7.2.3 Procedures to be followed if a bite occurs*

There are several important measures to be put in place for dealing with a bite (*48*), as described below.

#### 7.2.3.1 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.

#### 7.2.3.2 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 must be provided with the necessary materials (a number of crepe bandages, 10 cm wide × 4.5 m long, and splints). 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.

#### 7.2.3.3 Hospital admission

As a precaution, all victims of bites, scratches by snakes' fangs or teeth, and those in whom venom has entered the eye 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.

If, as highly recommended, the appropriate antivenom is stocked by the snake farm, a supply should 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 (e.g. anti-tetanus serum), and known allergies.

An occupational hazard of snake handlers is the sensitization to venom proteins. Two out of 12 snakebites resulted in venom-anaphylaxis in a venom production plant (*47*). Hypersensitivity is usually acquired by mucosal contact with aerosolized lyophilized venom. Important early evidence of evolving sensitization is sneezing, coughing, wheezing, itching of the eyes or weeping when 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 the emergency drugs cupboard.

#### 7.2.3.4 Medico-legal and health insurance aspects

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

### 7.3 Main recommendations

- **Well-managed snake farms 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 preclinical 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 milking, 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 of snake species, the venoms used for immunization should be collected from a large number of individuals (generally at least 20–50) collected from various regions covering the entire geographical distribution of the particular venomous snake species.**

- **Venom producers should follow the following recommendations rigorously 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 should be established and applied in cases where staff are bitten or have venom spat in their eyes.**
  - **Venom should not be milked from sick animals, which should be quarantined.**
  - **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 dessication 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.**

# 8 QUALITY CONTROL OF VENOMS

## 8.1 Records and traceability

It is critical to identify accurately the species (and the 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 bio-geographical origin of each snake should be specified, since differences in venom composition may occur between different populations of the same species or subspecies (*32–34*, *49*). 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 specificities of the venom (e.g. identification of all the snakes used, the species, subspecies and biogeographical origin, feeding, health care, date of each milking and quantity of venom produced). This information should be made available upon request to any auditor or control authority.

In the case of long-term storage, venoms could be regularly re-dried by dessication to ensure minimum water content, as this is critical to their long-term stability.

## 8.2 National reference materials

The quality of snake venoms used as a reference standard by quality control laboratories and national regulatory authorities is crucial.

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

Establishing reference venoms ensures that the antivenoms produced will be tested against the relevant venoms in the specific countries or regions.

Venom batches may be prepared following the procedure outlined in section 7. Whatever their origin, the snakes used for these reference standards should be accurately authenticated by a qualified person (species, subspecies) and the place of capture recorded.

It is the responsibility of the venom producer to provide clear information on 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.

## 8.3 Characterization of venom batches

In addition to the certificate mentioning 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, 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;
  - scans or pictures of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (in reducing and non-reducing conditions);
  - size-exclusion chromatographic profiles (e.g. high-performance liquid chromatography (HPLC));
- Enzymatic and toxicological activities of the venoms:
  - e.g. median lethal dose, $LD_{50}$.

If the venom producer is not able to perform these determinations, they can be subcontracted or, 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 system.

## 8.4 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 prepared or designed.**

- **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, should be made available by the venom supplier to the antivenom manufacturer as well as to the 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 and data recorded for traceability, including: the protein concentration per g (or mg), an assessment of biochemical or biological activity, scans or pictures from SDS–PAGE (in reducing and non-reducing conditions), and/or size-exclusion chromatographic profiles of the venom. This information has proved useful to confirm the origin and the integrity of the venom preparation.**

# 9 OVERVIEW OF THE PRODUCTION PROCESS OF ANTIVENOMS

Antivenoms are obtained following a complex production process (Figure 1), which involves several steps critical to quality, safety and efficacy. These steps are summarized below:

- Collection of venoms from individual venomous snakes that should be well identified and confirmed to be in good health. They should be representative of the region(s) where the resulting antivenom immunoglobulins are intended to be used.
- Milking of the selected snakes to prepare representative mixtures of venoms.
- Preparation of the venom(s) mixtures used for the programme of immunization 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 the antivenom immunoglobulins.
- 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 snakes used to prepare the venoms to immunize the animals are prevalent.

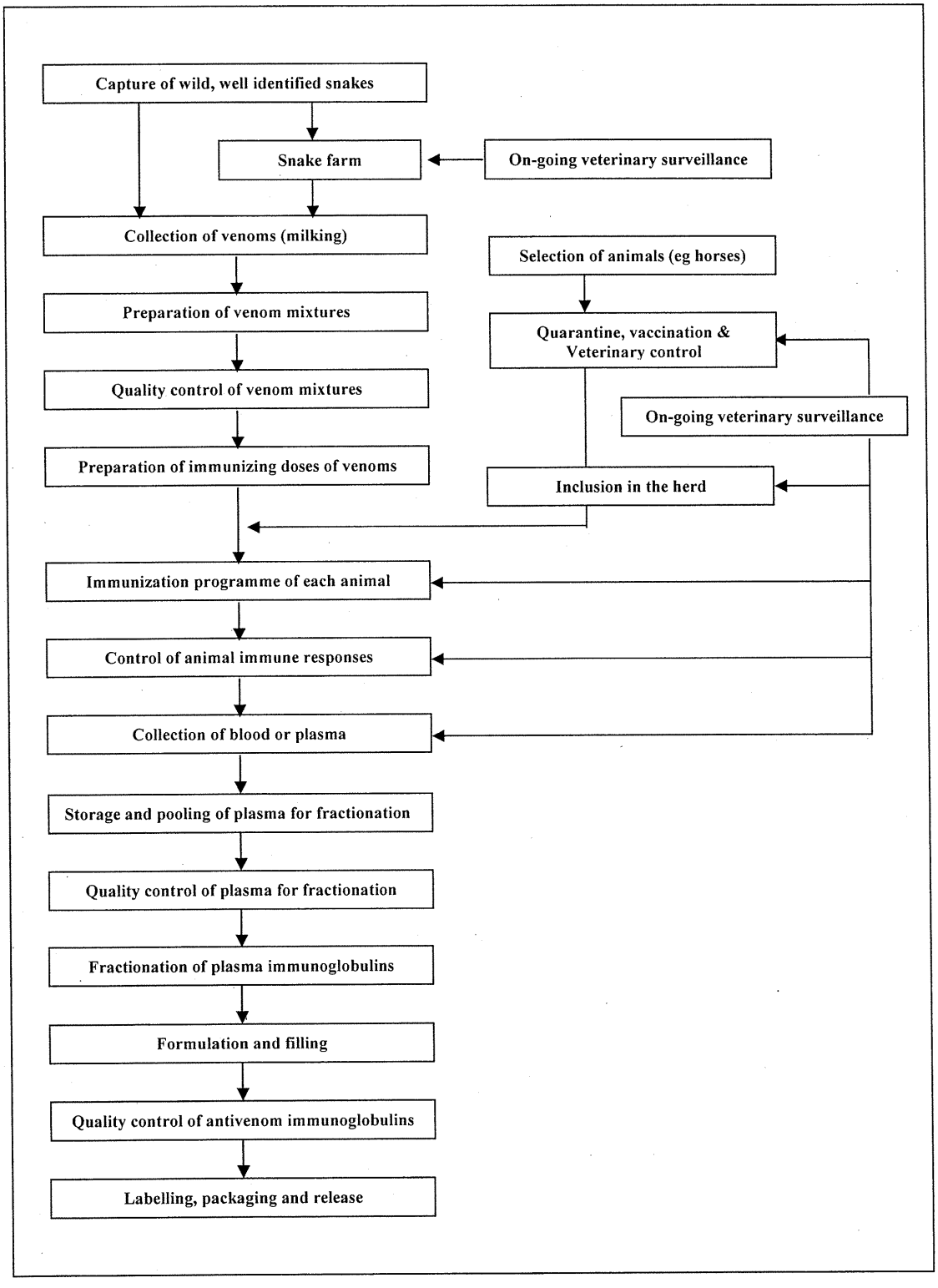


Figure 1
**General manufacturing process of antivenoms**

# 10 SELECTION AND VETERINARY HEALTH CARE OF ANIMALS USED FOR PRODUCTION OF ANTIVENOMS

## 10.1 Quarantine period

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, is from 6 to 12 weeks), depending upon the source of the animal, during which an appropriate veterinarian assessment is performed to ensure its suitability for the programme.

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 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 preferred because they yield larger individual volumes of blood.

## 10.2 Veterinary surveillance and vaccinations

The veterinary examination may include serological testing for the most prevalent infectious diseases for that type of animal in that particular geographical location.

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 programme to eliminate gut helminths and other locally prevalent parasites.

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

## 10.3 Animal health surveillance after inclusion in the herd

After the quarantine period, if the animal is in good health according to a veterinary check-up, 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 and maintenance should be clearly specified in the standard operating procedure.

During the time an animal is used for immunization aimed at antivenom production, careful veterinary surveillance should be maintained, including continued vaccination regimes, and the performance of regular clinical examinations, together with clinical laboratory tests such as haemogram, clotting tests and other tests associated with the possible clinical effects of venoms (*50*).

Possible anaemia, resulting from excessive volume or frequency of bleeding (when erythrocytes 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 immunization programmes to allow it to receive proper attention 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 sort 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[1]. Animals should have appropriate physical exercise. 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 adequate level of nutrients.

As a consequence of immunization with venoms (see section 11) 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 complete Freund's adjuvant are used. All injections should be given under aseptic conditions. There should be a limit to the total volume and dose of venom injected at a single site. Infected or ulcerated areas should not be used again until they have fully healed. 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.

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 GMP principles 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, unless the animal has been used in the preparation of a monospecific antivenom that is included into a polyspecific preparation, or if it was used for the production of other animal-derived antisera (e.g. anti-rabies, anti-tetanus, or anti-botulism).

### 10.4 Main recommendations

- **Animals intended for antivenom production programmes should be identified to ensure full traceability and health surveillance.**
- **Animals should go through a quarantine period of 6–12 weeks during which they are submitted to veterinary scrutiny and are vaccinated against and treated for parasites.**
- **Following the quarantine period, they are introduced into the immunization programme. Animals should be appropriately housed, fed, and managed according to the highest veterinary and ethical standards.**

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

- **During immunization, the clinical status of each animal must be followed by a veterinarian through clinical and laboratory assessments. If an animal develops signs of disease, it should be temporarily separated from the immunization programme to receive appropriate treatment. Particular care must be paid to the local lesions that develop at the site of venom injections.**
- **The immune response to venoms of each animal 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 each drug. In the case of vaccination, this withdrawal period should not be shorter than 1 month.**

# 11 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 components in the immunogenic toxins. To achieve this goal, the following considerations are important:

- Venom(s) used should be prepared as described in section 7, 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.

## 11.1 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) (*51*, *52*). 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 (*53*). 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. However, increasing concern about prion

diseases may limit the use of the sheep as an animal 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 10). When sheep or goats are to be used, manufacturers should comply with regulations to minimize the risk of transmissible spongiform encephalopathies to humans, such as the WHO Guidelines on tissue infectivity distribution in transmissible spongiform encephalopathies (*54*).

### 11.2 Venoms used for immunization

Venoms used as immunogens in antivenom production are chosen based on criteria discussed in section 6. Priority should be given to venoms from snakes responsible for frequent envenomings. The quality, quantity, and biological variation of venoms are important considerations (see sections 7 and 8).

### 11.3 Preparation of venom doses

Venom doses used for the immunization of animals should be prepared carefully in a clean environment, with 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 within established expiry periods. 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, labelled and stored appropriately (e.g. refrigerated, frozen at –15 to –20 °C, or deep frozen at –70 °C) for a short time (less than 1 month). However, it is recommended that venoms used for immunization be freshly prepared at the time of use.

All the equipment used for venom storage (freezers and refrigerators) and preparation (e.g. 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, i.e. 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. Transport of venom solutions to the facilities where animals are going to be injected should be done in a safe manner.

Care should be taken to avoid accidents that may result in envenoming of the persons preparing the venom solutions. Protective equipment (e.g. eyewear, gloves and gowns) should be worn by personnel preparing venom solutions. 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.

### 11.4 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 (*55*). 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 well-emulsified in an adjuvant such as Freund's complete or incomplete adjuvants.

### 11.5 Immunological adjuvants

Various types of immunological adjuvants have been tested, for example, Freund's complete and incomplete adjuvants, aluminium salts (hydroxide and phosphate), bentonite and liposomes (*56*). 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. Freund's incomplete adjuvant (FIA) contains mineral oil and an emulsifier. Freund's complete adjuvant (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 has been noted that the granuloma caused by FCA is due to injection of a large volume (5–10 ml) of the emulsified immunogen at 1 or 2 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 of injection, granuloma formation may be avoided.

### 11.6 Preparation of immunogen in adjuvants

To minimize infection at the immunization sites, all manipulations should be carried out under aseptic conditions. Venom solutions are prepared in distilled water or phosphate-buffered saline solution (PBS) 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 1.

**Box 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 °C. 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 remains stable when dropped on the surface of cold water. The highly viscous emulsion is then transferred into a sterile 50-ml glass 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 is then transferred from the 50-ml syringe into a 1-ml or 2-ml glass syringe. With the use of an 18G–20G needle, the water-in-oil emulsion is ready for subcutaneous injection.

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 needed before their injection.

When the immunogen is prepared in $Al(OH)_3$ (aluminium hydroxide) or $Al(PO)_4$ (aluminium phosphate), 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.

### 11.7 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 (Figure 2) should be in areas close to major lymph nodes, preferably on the animal's neck and back, while the route of injection should be subcutaneous so as to recruit a large number of antigen presenting cells and consequently resulting in 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 (*57*, *58*). An example of immunization of a horse using venom emulsified in FCA is described in Box 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 can be beneficial. Such isolated fractions can then be used for immunization.

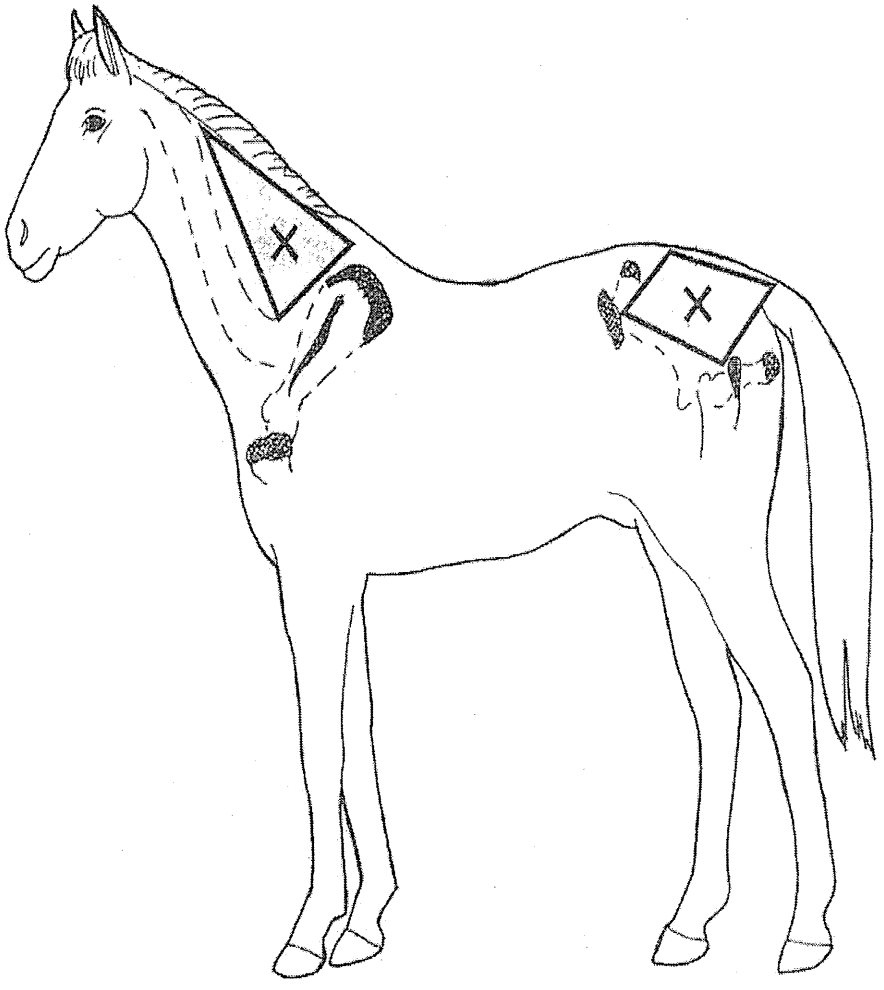

**Figure 2**
Recommended areas of immunization in horses