- Another approach is to combine antivenoms in a single product either by (a) immunizing individual animals with the venom of a single species and then mixing the various hyperimmune plasmas for fractionation, or (b) mixing in appropriate quantity the respective purified antivenoms before formulation. Proceeding this way may facilitate the adjustment of the respective potencies of the various antivenom specificities in the mixture. However, in such "combined antivenoms", neutralising antibodies against all individual venoms will be proportionally diluted. Such dilution implies that higher antivenom dose would have to be infused to patients, likely increasing the risks of adverse reactions.

In some regions, it is possible to differentiate envenomings based on obvious distinct clinical effects: neurotoxicity, local tissue damage and/or haematological disturbances (hemorrhage 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.

In general, the production of one polyspecific antivenom is simpler and less expensive than that of several different monospecific antivenoms. Polyspecific antivenoms have the additional advantage of simplicity of distribution and supply to different parts of a country. Clinical indications for their use are more straightforward since identification of the responsible snake species is less important. 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.

Polyspecific antivenoms resulting from the immunization of animals by a mixture of venoms offer significant clinical advantages and their production should be encouraged, whenever technically possible.

6.3 Main recommendations

- **When selecting antivenoms to use in their countries, National Health Authorities should first obtain and consider the information on the local snake 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 country or region. In general, polyspecific antivenoms are more convenient to use than monospecific antivenoms and simplify the selection of product for the treatment of envenomings.**
- **The preparation of polyspecific antivenoms obtained by immunizing animals with a mixture of venoms is preferred, when possible, to the mixture of individual monospecific antivenoms**
- **Manufacturers seeking marketing authorization of antivenoms in a given country should provide experimental evidence from preclinical testing that the product exhibits a neutralization capacity of local venoms (see Section 17).**

7 SNAKE VENOMS PREPARATION AND STORAGE

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 of critical importance, and their preparation should follow the GMP recommendations that are mentioned below.

Venoms used for antivenom manufacture should be representative of the snake population living in the area where the antivenom is used. In order to take account of the variability in venom composition of an individual species (Saravia et al., 2002; Faure and Bon, 1987; Creer et al., 2003), it is imperative that the venom of an adequate number of snakes (generally not less than 20 to 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 antivenoms is designed 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 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 recommendations is also strongly recommended:

- Rapid freezing of the venom after collection,
- Lyophilization of the venom during storage,[1] and
- Confirmation of batch-to-batch similarity of venom of the same origin.

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

7.1 Preparation of snake venoms for immunization

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

7.1.1 Quarantine of snakes

All new accessions should be quarantined for at least two 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, that should be eliminated using broad-spectrum antiparasitic drugs, as well as possible infections, in particular transmissible infections (Reichenbach-Klinke and Elkan 1965; Cooper and Jackson 1981; Frye 1991). Some viruses can be transmitted between different species (for example from *Bothrops* spp. to *Crotalus* spp.). When handling snakes, the risk of infection with human mosquito-borne viruses such as Japanese encephalitis should be prevented, since arbovirus infections in some snakes has been reported (Shortridge et al., 1974).

Sick snakes should be treated and their quarantine extended for two 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 four weeks following the end of the treatment. When housed in good conditions, adult snakes collected in nature can live in a snake farm for 10 years or more.

7.1.2 Maintenance of captive snakes for venom production

Ideally, a single species should be kept in each room to avoid mixing up with differente venoms. 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. 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-borne 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 sacrificing safety or allowing snakes to escape. 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 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 (e.g. 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 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 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 carefully cleaned at least each week. Access should be guarded by a tray containing an antiseptic which is placed on the floor at the entrance so that the footware 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 (water pipes, drainage conduits, ventilation entrances and exits etc.) should be protected by grills 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. 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, providing that they have similar living requirements (temperature, humidity).

Under favourable housing and climatic conditions and if left undisturbed, snakes will reproduce in captivity (Gans and Gans, 1984). Animals should be mated only with specimens from the same species, subspecies and local origin (Mitchell, 2004; Chanhome et al., 2001). Sexing can be difficult but is helped by the use of intra-cloacal probes. Male and 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 difference in the venom composition of adult and juvenile snakes had been reported, as in the case of *Bothrops* and *Crotalus* species (Gutiérrez et al., 1980; Furtado et al., 1991; Saravia et al., 2002; Alape-Girón et

al., 2008), 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, varying from twice per week to once per moth. 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, and constitute useful records related to the venom lots produced. Water should be provided in the milking room via 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, storage room for conservation of the venom produced, control laboratories and administrative rooms.

The cage cleaning rooms should be large enough for the storage of all the cages that are being cleaned and sanitized. Furthermore it is appropriate 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 where 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 be achieved in the same rooms, because of the stress induced by the rats on the mice. If snake reproduction is carried on in the farm, egg incubators, and special rooms for newborns and juveniles, are required. Their food requirements should take into account that the diets of young specimens might differ from those of adults (for instance, frogs and tadpoles are preferred instead of rodents in some species).

When possible, it is useful to have a small laboratory for performing quality control of the venoms (see Section 8). A space for repairing broken equipments and for miscellaneous other purposes is also required. The administrative area should be large enough and adequately equipped with computer facilities, so that the traceability requirements needed for venom production can be accomplished. The whole venom production facility should be made secure against unauthorised intrusion.

7.1.4 Snake milking for venom production

Specific safety consideration for operators should be applied for snake milking (see Section 7.2). Also GMP principles should be followed.

7.1.4.1 Venom collection in snake farms

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

Handling equipment should be appropriate for the particular species of snake to cause the least stress and should be familiar to and afford safety to the operator. The snake is 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. The use of short acting general anaesthesia during milking should be seriously considered (e.g. inhaled sevofluorane/sevoflurane, halothane or even CO_2) as it reduces the risk of accidents both to the snake and the snake-handler. Cooling 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 siliconised containers might be considered to minimise venom attaching to the container surface. While a brief electric 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 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 (Kurnik et al., 1999). 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 and require use of capillary tubes or similar 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), pentastomids escaping from the snake's respiratory tract and for areas of adherent dead skin and opercular scales over the snakes' 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 one hour from collection. For venoms with high proteolytic activity, this can be achieved by pouring the collected venom, for example, every ten minutes, or at least every 30 minutes, into a vial maintained at low temperature (ideally at -70 to -80°C, but, if not possible, at -20 to -40 °C) before another snake from the same group is milked. Centrifugation of freshly collected venom is recommended, since it removes cellular debris.

It is crucial to carefully 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. In order to obtain large venom batches for antivenom preparation, one approach is to use the same vial during 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 low temperature (either at -20 °C, or at 4°C) in a well-sealed flask, precisely identified with a number, up to delivery. However, some producers use an alternative system, keeping venom at 20-25°C in a dessicator. Once freeze-dried (or dessicated), venom batches should be divided in aliquots of defined amounts as required for production needs, adequately labelled and stored, since venom samples stored in large amounts in a single vial may be affected by repeated cycles of thawing and freezing. 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, protective clothing, a mask as well as vinyl gloves are 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 minimise cross-contamination. Likewise, equipment requiring calibration, such as

freezers, balances and freeze-driers, should be calibrated as per a defined schedule well established in standard operating procedures.

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 the 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. At anytime they may milk approximately from 50 to more than a hundred snakes; usually these are snakes of smaller size e.g. *Echis* species.

If venom collection from the wild snakes is necessary, most of the steps and safety procedures which are followed in milking captive snakes should be adhered as far as possible with modification for the field conditions. The team which goes to the field for collection also should include a herpertologist or zoologist who is able to help and confirm the identity of the snakes. Sick snakes, injured snakes and gravid females should be excluded from milking. 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. During milking, protective clothing, a mask as well as vinyl gloves are recommended to prevent any accidents or infections, as for collection in 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 antivenom administration 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, in order 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. (2006). At a commercial venom production plant in Uberlândia, Brazil between 1981 and 1999, 25 technicians performed 370,768 venom extractions from *Bothrops moojeni*. There were 12 bites, 10 with envenoming and one case of venom being squirted into the eye (Nishioka et al., 2000).

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 last every two hours, for a resting period before restarting the process.

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

7.2.2 Clothing and venom manipulation

Protective clothing should include 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 lyophilised 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 sensitise them to the venom. Appropriate gowning must be used when handling lyophilised venom, in order to avoid contact 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 as described below (Warrell, 2005a):

7.2.3.1 Procedures and alarms

Clearly defined, posted, 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 and available, in print form, adjacent to each case. Immediate application of pressure-immobilisation may be appropriate for bites by rapidly neurotoxic elapids. However, the technique is not easy and, if they are to use the method properly, staff will need extensive training (the technique is not easy) and should be provided with the necessary materials (a number of 10 cm wide 4.5 m long crepe bandages 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 pre-arranged transport for medical assessment. It may be helpful to remove from the cage and take 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 administer is ensured.

If, as it is 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 in a commercial venom production plant in Brazil resulted in venom-anaphylaxis (Nishioka et al., 2000). Hypersensitivity is usually acquired by mucosal contact with aerosolised lyophilised 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 the employed and requires their formal attention.

7.3 Main recommendations

- **The quality of snake venoms used for animal immunization, for the development of national/regional venom reference preparations or as material for preclinical assessment of antivenom neutralization efficacy 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 the production of antivenom therapeutic 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 bio-geographical distribution of the particular venomous snake species.**
- **Venom producers should follow rigorously 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**
    - **Appropriate housing, feeding, and handling of snakes according to veterinary and ethical standards, and following documented protocols**
    - **Adequate training of personnel involved in venom production in all procedures, and implementation of health and safety measures**
    - **Establishment and application of formal guidelines and procedures in case staff are bitten or have venom spat in their eyes**
    - **Absence of milking of venom from sick animals, which should be quarantined,**
    - **Full traceability of each venom batch**
    - **Freezing of venoms as soon as possible after collection, and at least within one hour**
    - **Freeze-drying or dessication of the venoms under conditions that ensure stability for long-term storage**
    - **Confirmation of batch-to-batch consistency of venoms of the same origin.**

8 QUALITY CONTROL OF VENOMS

8.1 Snakes origin, traceability, pool size

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

Internationally recognized scientific names should be used and the bio-geographical origin of each snake should be specified, since large differences in venom composition have been noticed between snakes of the same species and subspecies from different regions (Wüster and McCarthy, 1996; Saravia et al., 2002; Faure and Bon, 1987; Creer et al., 2003). Venom producers can enlist the help of academic zoologists who have appropriate skill and experience.

Data about each snake in the farm (identified by a "bar-code"), including feeding, health care and the quantity of venom it produces should be stored in a computer system. This allows venom lots to be traced. It is extremely important to carefully identify the vial where the venom has been collected with an appropriate reference number ("bar-code") that allows the identification of all the snakes used, the day of the milking, the name of the operator and any other relevant information. The same vial may be used for milking the same snake over several months or, even better, over one year, to take into account of seasonal variations in venom composition, provided that the cold chain is never broken. At the end of the collection period, the venom vial is freeze-dried (or dessicated, if stable) and kept in a well-sealed flask up to the time it is delivered. It is precisely identified with a number, and a chart containing all the information required for traceability. The residual moisture content of the venoms should be low (typically less than 3%) to ensure long-term stability.

Data associated to each numbered venom batch should allow the identification of all the snakes used, the species, subspecies and bio-geographical origin, the date of each milking and the name of the milking operator. This information should be provided by the venom producer upon delivery of each venom batch and will be a fundamental part within the quality assurance procedures of the antivenom manufacture.

8.2 National reference materials

The quality of snake venoms used as a reference standard by quality control laboratories and national regulatory authorities is very critical. National reference venoms materials should be prepared as described above (see Section 7). Due to the large variations in venom composition even within a single species it is recommended that national reference venoms should be

established. Regional reference materials could be used when countries would share similar snakes venomous distribution. Establishing reference venom materials ensure that antivenoms produced will be tested against the relevant venoms in the specific countries or regions.

Venom batches may be prepared following the procedure mentioned in Section 7. Whatever the origin, the snakes used need to be accurately certified by a qualified person (species, sub-species) as well as the place of capture of the animals. Venoms from snakes of a single region should be selected for each venom batch).

It is the responsibility of the venom producer to clearly provide information on the species, the sub-species and the local origin of the venom batches prepared for production, quality control and preclinical studies. This information should be included in the technical dossier supporting the marketing authorization of any antivenoms.

8.3 Characterization of venom batches

In addition to the certificate mentioning the scientific name of the snake species (and sub-species, if any), the geographical origin and the number of animals used for preparing the batch, the date of collection of the venom, etc., additional biochemical and biological information should also be provided for each venom batch. This information may include:

- Protein concentration per g (or mg)
- Scans or pictures of SDS-PAGE (in reducing and non reducing conditions)
- Size-exclusion chromatographic profiles (e.g. HPLC or FPLC)
- Enzymatic and toxicological activities of the venoms (e.g. Median Lethal Dose, LD_{50}).

If the venom producer would not be able to perform these determinations, they can be sub-contracted or, alternatively, depending on the agreement, the antivenom manufacture can perform those assays to confirm compliance of venoms specifications as part of the quality control of the raw material.

8.4 Main recommendations

- **Quality control of snake venoms is essential to give 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 possibly occurring during the preparation process.**
- **For each venom batch, a certificate mentioning the scientific names of the snake species (and subs-species, if any), their geographical origin and the number of**

animals used for collecting the batch, the date of collection of the venom, etc., 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 controls should be performed in each venom sample and data recorded for traceability: the protein concentration per g (or mg) of freeze-dried venom, an assessment of biochemical or biological activity, scans or pictures of SDS-PAGE (in reducing and non reducing conditions), and/or size-exclusion chromatographic profiles of venom sample. This information has proved useful to confirm the origin of the venom and the absence of proteolytic degradation.**

9 OVERVIEW OF THE PRODUCTION PROCESS OF ANTIVENOMS

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

1. Collection of venoms from venomous snake individuals 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.
2. Milking of the selected snakes in order to prepare representative mixtures of venoms.
3. Preparation of the venom(s) mixtures used for the immunization programme of animals (most often horses). Animals should be selected and controlled carefully, and subjected to continuous health surveillance.
4. Collection of blood/plasma from the immunized animals, once the immune-response to the immunizing venom mixture has yielded satisfactory antibody levels.
5. Preparation of the pool of plasma for fractionation.
6. Fractionation of the plasma to extract the antivenom immunoglobulins.
7. Formulation of the bulk antivenom immunoglobulins and aseptic filling
8. Quality control tests, including potency assessment by in vivo assay
9. Labelling, boxing, and release
10. Distribution in the region(s) where snakes used to prepare the venoms to immunize the animals are prevalent

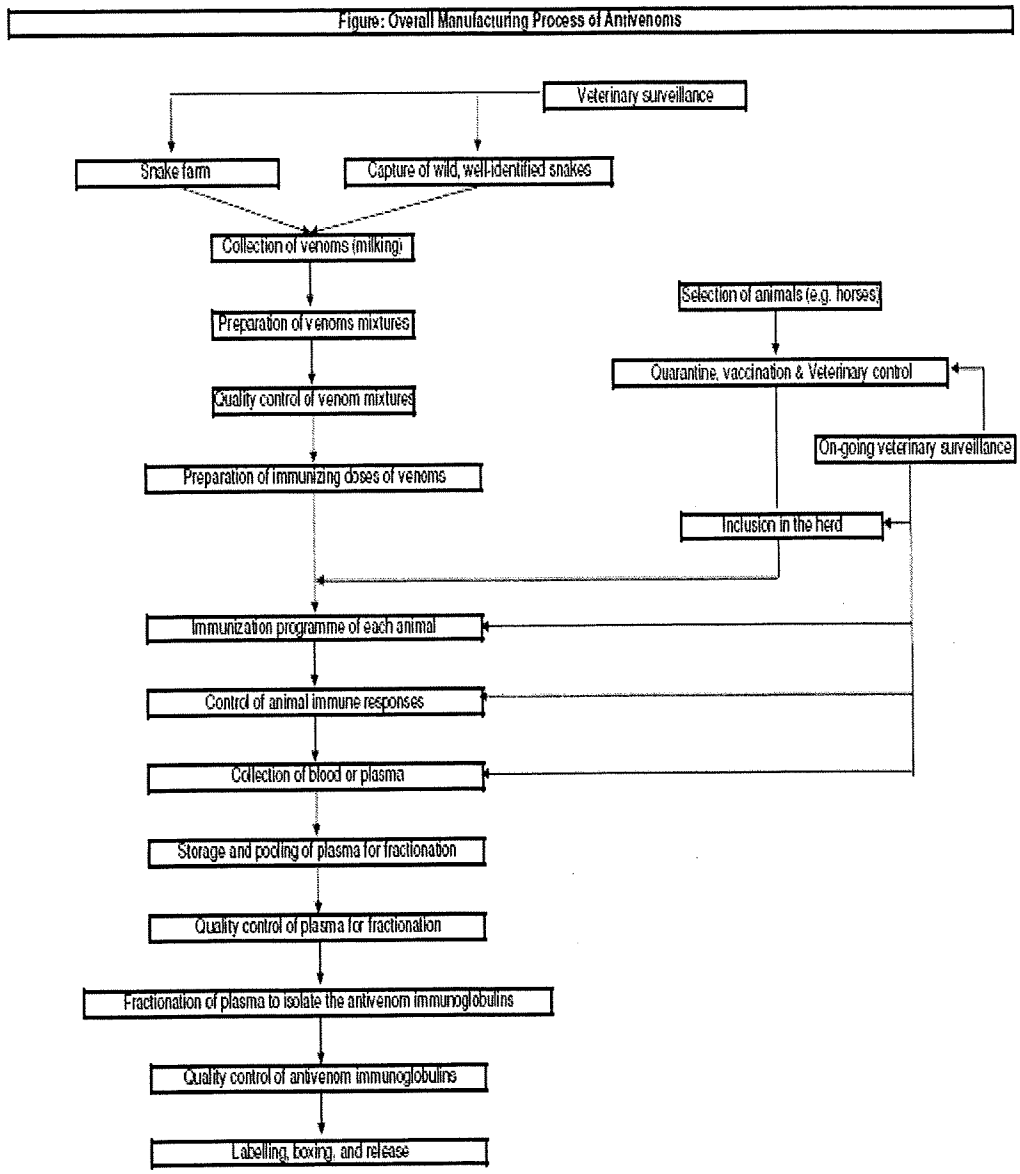

Figure 1: Overall manufacturing process of antivenoms

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

10.1 Quarantine period

Before an animal is introduced into a production programme, it should be subjected to a period of quarantine lasting 6 to 12 weeks, depending upon the source of the animals, during which an appropriate veterinary assessment is performed to ensure its suitability for the programme.

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

In the case of horses, animals between 3 and 10 years are usually included in an immunization program, but in some cases older animals may also be suitable as long as they exhibit satisfactory immune response from 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 rabies and, possibly, other endemic diseases, such as 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 in regular contacts 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 animals are in good health conditions according to a veterinary check-up, and, 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 immunization programmes for antivenom production. In addition to the surveillance of 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 (Angulo et al., 1997).

Possible anaemia, resulting from excessive volume or frequency of bleeding (when erythrocytes are not re-infused to the animals after the whole blood bleeding session) should also be controlled.

The immune response against venom components should be followed throughout the immunization schedule, in order to detect when animals reach an acceptable antivenom titre. This response may be followed by in vivo potency assays of neutralization of lethality or by in vitro tests, such as enzyme immuno-assays (EIA) (provided that a correlation has been demonstrated between these tests and the in vivo potency tests).

Whenever an animal develops any manifestation of disease, it must be temporarily withdrawn from immunization programmes, in order to allow proper attention and treatment. Once the disease is controlled, the animal may return to the immunization programme after a suitable delay of usually 4 weeks. Also, whenever the animal has received an antibiotic, a live vaccine, or drugs there should be a withdrawal period of four weeks before the collection of blood for antivenom production. These delays are intended to ensure a clearance from the blood circulation.[1] Animals should have appropriate physical exercise. They should be adequately fed, ideally with a diet that includes both hay/grass or alternative plant material and concentrated food preparations containing vitamins including folic acid, iron and other mineral supplements. A 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 sterile conditions. There should be a limit to the total volume and dose of venom at a single injection site. Infected/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 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 program to another one, unless the animal has been used in the preparation on a monospecific antivenom that is included into a polyspecific

[1] In some legislations, animals used for production of plasma cannot be treated with penicillin or streptomycine

preparation, or if it was used for the production of other animal-derived antisera (anti-rabies, anti-tetanus, anti-botulism, etc.).

When an animal is withdrawn from the herd, it could be either kept within the horse farm or ensured to receive good care if sold.

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 to 12 weeks during which they are submitted to veterinary scrutiny, are vaccinated against and treated for parasites.**
- **Then, they are introduced into the immunization programme. Animals should be appropriately housed, fed, and managed according to the highest veterinary and ethical standards.**
- **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 be monitored during the immunization schedule.**
- **If an animal is receiving any sort of antibiotic, drug, or is vaccinated with live attenuated vaccines, it must be withdrawn from the immunization program for a period of four weeks before the collection of blood for antivenom production.**

11 IMMUNIZATION REGIMENS AND USE OF ADJUVANT

One of the most crucial steps in antivenom production involves the immunization of animal with venom(s) to produce a long-lasting and high titer antibody response against the lethal and other deleterious components in the immunogenic toxins. To achieve this goal, some important considerations should be made:

1. Venom(s) used should be prepared as described in Section 7, and should be in an optimal condition for inducing specific and neutralizing antibodies.
2. Immunogen and the immunization regimens used should not seriously affect the health status of the animal.

3. Preparation of immunogens and the immunization protocol should be technically simple and economical and use 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 the doses; selection of adjuvants; sites of immunization; 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 at various scales in antivenom production (horse, sheep, donkey goat, rabbit) or for experimental purposes (camel, llama, dog, hen) (Landon and Smith, 2003; Landon et al.,1995). However, the production of large volumes of antivenom from large animals such as horses 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, cost of maintenance, etc. Information in these Guidelines refers mostly to horse derived immunoglobulins.

The horse is the animal of choice for commercial antivenom production. They 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. Sheep have also been used as an alternative source for antivenom production because they are cheaper, easier to rise, can better tolerate oil-based adjuvant than horses, and their antibodies may be useful in patients hypersensitive to equine proteins. However, increasing concern about prion diseases may limit the use of 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 would be used, manufacturers should comply with regulations to minimize risk of transmissible spongiform encephalopathies to humans such as the WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (WHO TSE , 2006).

11.2 Venoms used for immunization

Venoms used as immunogens in antivenom production are chosen based on criteria discussed in Section 6. The 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 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 venoms lots used and the animals to be dosed should be recorded and the containers where the venom is dissolved should be appropriately identified. Ideally, the calculations and operations related to dose of venom to be used, dilutions, etc. require verification by a second person to ensure accuracy and to prevent errors that may lead to animal overdosing.

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 should be sterile-filtered, aliquoted, labelled and stored frozen at -15 to -20°C for a short period of time (less than a 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, refrigerators) and preparation (balances, etc.) 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, etc., should be pre-sterilised, single use disposable items. The siliconisation 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 (eyewear, gloves, gowns) should be worn by personnel preparing venom solutions. Procedures for cleaning up broken glass or plastic containers with venom should be prepared and the personnel should be trained to follow them.