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 freezedried 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:

o Taxonomic identity and geographical origin of each individual animal used for venom production should be known and recorded

o Appropriate housing, feeding, and handling of snakes according to veterinary and ethical standards, and following documented protocols

o Adequate training of personnel involved in venom production in all procedures, and implementation of health and safety measures

o Establishment and application of formal guidelines and procedures in case staff are bitten or have venom spat in their eyes

o Absence of milking of venom from sick animals, which should be quarantined,

o Full traceability of each venom batch

o Freezing of venoms as soon as possible after collection, and at least within one hour

o Freeze-drying or dessication of the venoms under conditions that ensure stability for long-term storage

o 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 freezedried (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, subspecies) 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 subspecies, 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 subcontracted 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 SDSPAGE (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.

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 adapted in order to decrease venom toxicity, for example, treatment with aldehydes (formaldehyde or glutaraldehyde), hypochlorite, UV or gamma radiation, heat, etc. However, in most cases, not only the toxic sites but also the antigenic sites of the toxins are destroyed after these treatments (Moroz-Perlmutter et al., 1963). 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 necessary if inoculation is made with small dose of venom well-emulsified in adjuvants like Freund's complete and incomplete adjuvants.

11.5 Immunological adjuvants

Various types of immunological adjuvants have been tested, for example, Freund's complete and incomplete adjuvants, aluminum salts (hydroxide and phosphate),

bentonite, liposomes etc. (Freitas et al., 1991). The choice of adjuvant is determined by its effectiveness, side effects, ease of preparation, especially in large scale, and cost. It may vary depending upon the type of venoms and following manufacturers experience. Freund's complete adjuvant (FCA), which contains mineral oil and *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 1-2 sites. The large granuloma formed usually ruptures, resulting in 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

In order to minimize infection at the immunization sites, all manipulations should be carried out under aseptic conditions. Venom solutions are prepared in water or phosphate-buffered saline solution (PBS) and filtered through 0.22 µm membrane. The venom solution is then mixed and/or emulsified with adjuvant, according to the instruction of the supplier. An example for the preparation of venom immunogen in FCA/FIA and aluminum salts is detailed in Box 1. Immunogen in FIA is prepared similarly as described above except that FIA is used in place of FCA. Both the FCA and FIA emulsified immunogens, if necessary, may be stored at 4° C for several weeks but re-emulsification is needed before their injection. When the immunogen is prepared in $Al(OH)_3$ (aluminum hydroxide) or $Al(PO)_4$ (aluminum phosphate), a sterile venom solution and a suspension of aluminum salts are mixed in a ratio of 1:3 (v/v) and homogenized. When using other adjuvants, the preparation of 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 such as soap, shaved and rubbed with 70% ethanol before venom immunogen injection. In general, the sites of immunization (Figure 2) should be made in areas close to major lymph nodes, e.g., 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 result in high antibody response. Some procedures call for small volume of injection at each site (50-200 μ l) so that the total surface areas of the immunogen droplets is maximized, enhancing the interaction with the antigen presenting cells and the immuneresponse (Pratanaphon et al.,1997: Chotwiwatthanakun et al., 2001).

Box 1: Example of preparation of venom immunogen in FCA,FIA and aluminum salts

Since FCA can cause severe irritation, precaution should be taken to avoid eye contact, 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 approx. 3,000 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 puncher removed. The puncher is then put onto the syringe to expel any air pocket inside. By means of a 3-way stopcock, the emulsion is then transferred from the 50 ml syringe into a 1 or 2 ml glass syringe. With the use of an 18G- 20G needle, the water-in-oil emulsion is ready for subcutaneous injection Figure 2: Recommended areas of immunization in horses An example of immunization of horse using venom emulsified in FCA is described in the box 2.

Box 2 Example of immunization of horse using FCA adjuvant

The primary immunization could be made with venom(s) mixed with Freund's complete adjuvant (FCA) as described in Box 1. The initial dose of each venom could be as low as 1-4 mg/horse with a total combined volume of injection of about 2 ml. The immunogen is filled in a 1 ml glass syringe with 18G needle as described above. Subcutaneous injections of 100-200 µl of immunogen are made at each site, up to as many as 8 to 12 sites, although some producers may use 3 to 4 injection sites only. The neck of the horse, supplied with extensive lymphatic vessels and large lymph nodes, is a preferred area for immunization. If inoculation is made on the lateral sides of the neck, the animal tends to rub itself against the stable causing skin blisters. Thus, injections should be made to the upper part (dorsal) of the neck close to the mane. About 4 - 6 injections can be made at each side of the neck. If injection at the rump is possible, 1-2 injections can be made in the area between the outer hip bone and the top of the thigh bone. The scratching of injected sites by animals can be partially alleviated by massaging the injection site after venom injection to disperse the dose material. Immunization using Freund's complete adjuvant is usually made only once; repeated use of this adjuvant may in most cases cause serious reactions which can affect the horse's health. After 2 weeks, the horses should receive a booster injection with the same venom(s) well emulsified in Freund's incomplete adjuvant. Similar volume and areas of injection to those described above can be made. Subsequent booster immunizations at 2 week intervals can be made with higher doses (5-10 mg) of venom(s) in saline or mixed with aluminum salts or any other adjuvant selected. In this case, subcutaneous injections of 1 ml of immunogen at each site in a total of 4 sites are recommended. Blood (10-20 ml) should be drawn before each immunization. Serum or plasma is prepared and EIA (enzyme immunoassay) titres and/or lethality potency are determined. When the EIA titres reach a plateau, usually about 8-10 weeks after the primary immunization, an in vivo potency assay may be performed to confirm that the horse could be bled. After bleeding for antivenom production, the horses are allowed 4 to 8 weeks rest, depending on their physical condition. After the rest period, a new round of immunization can be made as described above but without the use of Freund's complete adjuvant. 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 m ain 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.

11.8 Traceability of the immunization process

The traceability of the immunization process is very critical for the quality control of the produced antivenoms and it should be performed very accurately. Each immunized animal should be identified by their code number (see Section 10). The details of each immunization steps should be recorded: precisely: date of immunization, batch(es) of venom(s) used with its (their) reference number(s) (see Section 8), venom dose(s), adjuvant and/or salt used, name of the veterinary and supporting staff in charge of the immunization, eventual reaction and/or sickness, etc. The antivenom titer of the immunized animals should be followed during all the immunization procedure either in vitro (enzyme immunoassays-EIA) during the immunization phase, or in vivo, by neutralization potency assays of lethality when the immunization plateau is reached or before each blood collection. Each plasma batch should be assigned a unique reference number (e.g. "bar-code") which should allow identifying it unambiguously. Information such as the date of collection, the number of the immunized donor animal, the venom(s) and its (their) reference number(s) used for immunization, etc should be linked to each plasma batch. Computer-based databases are very useful to record properly these data which are of a major importance for the traceability of the produced antivenoms. Standard procedures should be used to protect the integrity of data stored on a computer, including regular, frequent backup, protection from unauthorized access, and storing back-up copies securely off-site.

11.9 Main recommendations

- Venom solutions should be prepared in such a way that minimizes proteolytic digestion and denaturation of the venom proteins. Venom solution should be prepared under aseptic conditions to avoid infection at the injection sites.

- The type of adjuvant used is selected based on the effectiveness, side effects, ease of preparation and cost.

- Primary immunization should be made by subcutaneous injections in small volumes at multiple sites close to the animal's lymphatic system to favor the recruitment of antigen presenting cells and involving anatomically different groups of lymph nodes for antibody production

- Subsequent booster injections can be made using venom immunogen doses, volumes and intervals depending on the type of adjuvants used, until the antivenom titer reaches a plateau or a pre-established minimum accepted titer.

- After collection of blood for antivenom production, animals should have a resting period of 4-8 weeks After this, a new round of immunization can be performed as above without the use of Freund's complete adjuvant.

- The traceability of each immunized animals, every step of the immunization procedure and every plasma batch collected is very important for the quality control of the produced antivenoms and is the responsibility of the antivenom manufacturer. –

12 COLLECTION AND CONTROL OF ANIMAL PLASMA FOR FRACTIONATION

Historically, serum separated from the blood of hyperimmunized horses was the basis of "antivenin serum-therapy", but today plasma is used, almost exclusively, as the starting material and undergoes a fractionation process for the separation of purified antivenoms. Thus "antivenom immunoglobulins" is the preferred term, rather than

"anti-snakebite serum" or "antiserum" which are imprecise and confusing terms that refer to a crude therapeutic preparation. The use of plasma as a starting material is preferred to serum largely because erythrocytes can be returned to the animal, thus preventing anaemia and hypovolaemia in the donor animal and allowing more frequent bleeding. Some laboratories have found that using plasma provides higher recovery of antibodies per donation and it is less contaminated with haemoglobin. Separation of plasma from anticoagulated blood is much more rapid than separation of serum from clotted blood. Plasma for fractionation can be obtained either from the collection of whole blood or by apheresis procedure.

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

When an immunized animal has reached an acceptable antivenom antibody response, it can be bled. Before bleeding is performed, the animals should be evaluated by a veterinary and declared healthy. Animals having evidence of clinical deterioration, such as low hemoglobin concentration, low protein content, or evidence of infections, should not be bled. Moreover, as already indicated in Section 10, if the animals have received antibiotics or any other therapeutic drugs or live vaccines during the last four weeks, they should not be bled either. It is very important to assure, as much as may be ascertained, that the animal is free of pathogens and that any drugs the animal may have received for treatment.has been cleared from the blood.

12.2 Blood/plasma collection premises

The bleeding of animals should be performed in designated rooms dedicated to this activity. Some producers may design the bleeding rooms so that they can be closed, if needed, during the bleeding sessions, but this is not general practice. Rooms should be extensively washed and cleaned before and after each bleeding session and their design should facilitate such cleaning procedures, which should be clearly established. Animals need to be made as safe and comfortable as possible during bleeding to minimise the chance of injury to the animal or handlers. Individual animals should be confined in circumstances that reduce the stress as much as possible. It is recommended that these rooms allow the simultaneous bleeding of various horses in order to reduce the time required for this operation as well as the stress.

12.3 Blood/plasma collection session

Animals are bled by venipuncture from the external jugular vein. The area surrounding the venipuncture site should be shaved before bleeding and thoroughly cleaned and disinfected, using a non-expired disinfectant, and, depending on the type of disinfectant, allowed, to dry. The prepared area should not be touched before needle has been inserted. Prior to venipuncture the containers –either plastic bags, plastic bottles or glass bottles, should be pre-weighed and inspected for defects. Any abnormal discolouration suggests a defect and the container should not be used. The clinical condition of the animals being bled should be closely scrutinized during the time of bleeding and the days afterwards, and bleedings should be suspended in the event of any adverse effect to the animal. Animals showing signs of distress during the operation should have the collection operation terminated

12.4 Labelling and identification

The identity of the animal should be recorded immediately before venipuncture. Each bottle/bag of plasma should be labelled with details of animals from which plasma

comes from, specificity of antivenom, blood/plasma weight or volume, donation number, and date of collection.

12.4.1 Whole blood collection and storage

12.4.1.1 Collection

The volume of blood to be obtained depends on the species and size of the immunized animal. It is recommended that around 13 to 15 mL of blood per kilogram body weight are collected in one bleeding session. In the case of sheep, 0.5 L is a typical yield, whereas in the case of horses, the volume of blood may range between 3 to 6 L, depending on the size of the animal. Blood can be collected, ideally, in disposable plastic bags containing sterile citrate anticoagulant. Usually, the volume ratio of anticoagulant to blood is of 1 to 9. Use of double plastic bags containing anticoagulant is recommended to avoid bacterial contamination and for ease of use. When plastic bags are not available, disposable polypropylene plastic bottles, or sterilized glass bottles containing anticoagulant may be considered. When the bleeding is taking place, a constant flow of blood should be ensured. Blood should be gently and continuously mixed with the anticoagulant solution to ensure an homogeneous distribution of the anticoagulant, to avoid the risks of activation of the coagulation cascade and, therefore, avoid the formation of clots. The duration of a bleeding session is usually comprised between 30 and 45 minutes depending upon the weight of the animal and the total volume collected. Care should be taken to avoid contamination of the blood by exposing the needle to contaminated surfaces.

12.4.1.2 Storage

Whole blood: bags or bottles in which whole blood is collected should be appropriately cleaned and sanitized on their external surfaces. They should be put into a refrigerated room (2-8 °C) for the plasma and blood cells separation procedure. They should be stored up to 24 hours until the reinfusion of the red cells.

12.4.1.3 Separation of plasma from whole blood

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

12.4.1.4 Reinfusion of the erythrocytes

Blood cells, most specifically erythrocytes (red blood cells), should be separated from plasma by validated centrifugation or sedimentation procedures. It is highly recommended that erythrocyte suspensions should be returned to the horse. Erythrocyte reinfusion should take place within 24 hours after blood collection, after being suspended in sterile saline solution and warmed up to 35-37°C prior to infusion. Such procedure where whole blood is collected and erythrocytes are reinfused to the animal is commonly referred to as "manual apheresis". 12.4.2 Plasma collection by automatic apheresis and storage

12.4.2.1 Plasma collection

In some laboratories, plasmapheresis machines are used to perform automatic plasma collection. This has proved a useful investment in some facilities; it ensures that

the animal does not become hypovolemic and it reduces the risks of handling errors, in particular during re-infusion of the erythrocytes to the donor. Automatic apheresis plasma tends to be less contaminated by blood cells (red blood cells, leucocytes and platelets) and in the experience of some laboratories is easier to fractionate as filtration steps, in particular, are more readily performed, resulting in higher yields. In such procedures, whole blood is collected from the animal, mixed with anticoagulant, and passed through an automated cell separator. The plasma is separated from the cellular components of the blood, which are returned to the animal in a series of collection/separation and return cycles. The plasma is separated from the red blood cells by centrifugation or filtration, or a combination of both. The operational parameters of the plasmapheresis equipment are provided by the manufacturers of the equipment. In general, the anticoagulant is delivered at a rate yielding a specified ratio of anticoagulant to blood. The anticoagulant solutions used include AB16 (35.6 g sodium citrate; 12.6 g citric acid monohydrate; 51.0 g glucose monohydrate per 1 litre using water for injection) and ACDA (22.0 g sodium citrate; 8.0 g citric acid; 24.5 g dextrose monohydrate, per 1 litre using water for injection). The number of collection/separation and return cycles for each donor animal depends on the total volume of plasma that is to be harvested. For horses, the average volume of plasma collected may be about 6 liters per session. The number of cycles ranges from 10 to 20 depending upon the hematocrit of the horses. The collection process lasts for 1 to 4 hours. The apheresis equipment and apheresis procedures should be validated, maintained and serviced. Machine plasmapheresis can take hours and so animals can be fed during the operation

12.4.2.2 Plasma storage

Apheresis plasma: bags should be stored in a refrigerated room (2-8 °C) under the dark until the fractionation process starts. This storage room should be designed to allow a proper cleaning and sanitization.

12.5 Pooling

Plasma from individual animals should be mixed in sterile and sanitized bottles or plastic containers to prepare pools before fractionation. For traceability purpose, each plasma pool should be identified with a unique number (e.g. "bar-code") and the numbers of plasmas from individual animals used in the pool should be recorded precisely. Such pooling should be performed under aseptic conditions and pools should be adequately identified. Individual or pooled plasma should be stored at 2-8°C in a room devoted for this purpose. In order to assure the prevention of microbial contamination of plasma, preservatives (phenol or metacresol) can be added at a dose of less than 3g/l at this stage and kept during storage of plasma. In order to avoid the risk of contamination, it is recommended that individual or pooled plasma are not stored for long time intervals before fractionation, i.e. they should be fractionated as soon as possible after pooling. In the event that plasma should be stored for prolonged periods of time (for instance 6 months), the storage time and conditions should be validated to ensure that this does not have a detrimental impact on the quality of the plasma material, on the fractionation process, and on the quality, efficacy and stability of the antivenoms.

12.6 Control of plasma prior to fractionation

Before fractionation, pools of plasma should be checked for macroscopically-evident precipitates, gross haemolysis and bacterial contamination (sterility test or bioburden assay). The neutralizing potency of the starting plasma should be ensured so that the resulting antivenoms will be within potency specifications. Additional controls may include, when relevant, pyrogen test, endotoxins determination, and total protein content. Plasma pools should be discarded in case of bacterial contamination or if the neutralizing potency is below a minimum limit established by the producer. Cloudy plasma proved free of bacterial contamination may still be used for fractionation provided the fractionation process and product quality has been proven not be impaired. Grossly haemolysed plasma should not be used for fractionation.

12.7 Main recommendations

□ **When animals have developed an adequate immune response against venoms, and if they are in good health, they can be bled for antivenom production. Bleeding should be performed in enclosed rooms that should be kept scrupulously clean. Traceability of the donations should be ensured.**

□ **Plasma is preferred to serum as a source material. Animals should be bled from the external jugular vein. Plasma can be obtained either from whole blood or by automated plasmapheresis and using approved anticoagulants. Blood or plasma should ideally be collected into closed plastic bags. When this is not possible, glass or plastic bottles can be used, if they can be readily cleaned and sterilized.**

□ **Plasmapheresis is recommended using either automatic or manual procedures. When manual apheresis is used, blood cells should be sedimented, separated from the plasma, resuspended in saline solution and returned to the animals within 24 hours. Plasma separation should be performed in a designated room under aseptic conditions.**

□ **Bags or bottles containing plasma should be thoroughly cleaned on their external surfaces, adequately identified and stored in refrigerated rooms for further fractionation.**

□ **Plasma should be controlled prior to fractionation to establish compliance with relevant acceptance criteria for fractionation, in particular the neutralizing potency.**

□ **A special attention should be directed to ensuring traceability between individual animal donors and the plasma pool.**

13 PURIFICATION OF IMMUNOGLOBULINS AND IMMUNOGLOBULIN FRAGMENTS IN THE MANUFACTURE OF ANTIVENOMS

The purification of immunoglobulins and immunoglobulin fragments for the manufacture of antivenoms should aim at obtaining products of consistent safety and efficacy, manufactured following GMP principles. These preparations should have the following characteristics:

□ Effective in the neutralization of the targeted venoms,

□ High purity of the active substance,

□ Low content of protein aggregates,

□ Free of microbial contaminants, and

□ Low in endotoxins.

13.1 Good Manufacturing Practices

The quality and safety of antivenoms rely largely on the manufacturing procedures that

have an impact on the biological characteristics of the end-products. The fractionation process used to manufacture antivenoms should adhere to the general GMP recommendations developed for medicinal products. All operations should therefore be carried out in accordance with an appropriate and relevant system of Quality Assurance and GMP. This covers all stages leading to the finished antivenoms, from production of plasma (including animal selection and health control, production of venoms and immunization protocols, containers used for blood and plasma collection, and anticoagulant solutions, and quality controls methods) to purification, storage, transport, processing, quality control and delivery of the finished product. Of particular relevance is the control of microbiological risks, particulate and pyrogen contamination, and the existence of a documentation system that ensures the traceability of all production steps. In order to establish a satisfactory traceability of the antivenom produced, all the steps of the purification procedure used for the preparation of the antivenom batch should be recorded carefully in preestablished and approved batch record documents, and sampling should be made at established critical steps for in-process quality control tests. WHO Guidelines on Good Manufacturing Practices for medicinal products are available (2007d) and main principles on GMP for the manufacture of blood plasma products of human origin have also been published (WHO 2003; 2007e). These Guidelines can serve as a general guide for manufacturing practices used for preparing antivenoms derived from animal plasma. Useful specific reference in the field of antivenoms includes also the Note for Guidance on production and quality control of animal immunoglobulins and immunosera for human use (CPMP/BWP/3354/99) (CPMP, 2002).

13.2 Purification of the active substance

Antivenoms are prepared from the starting plasma pool using diverse methodologies to obtain one of the following active substances: (1) Intact IgG molecules, (2) $F(ab')_2$ fragments, or (3) Fab fragments. In general, fractionation procedures should not impair the neutralizing activity of antibodies, should yield a product of acceptable physicochemical characteristics and purity, with low content of protein aggregates, free of endotoxins, and should provide good recovery of antibody activity. The characteristics of a batch of plasma to be fractionated should be clearly established, and the methods used to purify the active substance and the in-process controls should be described in detail in SOPs.In the following sections, examples of basic protocols used for the production of IgG, $F(ab')_2$ and Fab antivenoms are described. Some additional methodologies introduced to further purify the active substance of antivenoms are also discussed. Variations in those manufacturing procedures have often been developed by individual fractionators and should be considered as acceptable when shown yield safe and effective for antivenoms preparations.

13.2.1 Purification of intact IgG antivenoms

13.2.1.1 Ammonium sulphate precipitation

In the past, some laboratories have utilized fractionation protocols based on salting-out procedures employing ammonium sulphate or sodium sulphate (Bolaños and Cerdas, 1980). Two precipitation steps are included using two different salt concentrations, in addition to the elimination of 'euglobulins' by precipitation in a diluted acidic solution. Such fractionation protocols generally leads to a low recovery of