antibodies (40-50 %) and to the formation of protein aggregates. The final product contains a relatively high content (>15 %) of contaminating proteins, such as albumin (Rojas et al., 1994). This impairs the safety of the product, since a high incidence of early adverse reactions has been described with such intact IgG antivenoms (Otero-Patiño et al., 1998; Otero et al., 1999).

*13.2.1.2 Caprylic acid precipitation*

The use of caprylic acid (octanoic acid) as a precipitating agent of proteins from animal sera has been described in the literature (Steinbuch and Audran, 1969). Several procedures for the purification of whole IgG antivenoms of good physicochemical profile and purity using caprylic acid precipitation of non-immunoglobulin proteins have been developed and are now used at production scale (Dos Santos et al., 1989; Rojas et al., 1994; Gutiérrez et al., 2005). Fig 3 describes a particular process where caprylic acid is added slowly to undiluted plasma, under constant stirring, to reach a concentration of 5 % (v/v) and pH 5.5. The mixture is stirred at 22-25 °C for a minimum of one hour. Precipitated proteins are then removed by filtration or centrifugation. The precipitate is discarded and the filtrate (when using filtration) or the supernatant (centrifugation) containing the immunoglobulins is then submitted to tangential flow diafiltration, to eliminate the residual caprylic acid and to concentrate the filtrate. Alternatively, elimination of residual caprylic acid can be achieved by dialysis. The immunoglobulin solution is then formulated by adding NaCl, an antimicrobial agent and any other excipient needed for formulation, such as protein stabilizers, and the pH is adjusted, generally to neutral values. Finally, the preparation is sterilized by filtration on 0.22 µm filters, and dispensed into final containers (vials or ampoules). Variations in this procedure have been introduced by various manufacturers, and include dilution of plasma, changes in caprylic acid concentration, pH, temperature, etc. Caprylic acid fractionation allows the production of antivenoms of relatively high purity and with a low protein aggregate content because the immunoglobulins are not precipitated. The yield may reach up to ~ 60-75% of the activity in the starting plasma, depending upon the details of the procedure and/or the equipment used. The efficacy and safety profiles of caprylic acidfractionated antivenom immunoglobulins have been demonstrated in clinical trials (Otero-Patiño et al., 1998; Otero et al., 1999). Fig 3: Example of one fractionation process of intact IgG antivenoms prepared by caprylic acid precipitation of non-immunoglobulin proteins

13.2.2 Purification of $F(ab')_2$ antivenoms

Many manufacturers follow the classical protocol for $F(ab')_2$ antivenom production of Pope (1939a, 1939b), with a number of recent modifications (Raw et al., 1991; Grandgeorge et al., 1996; Jones and Landon, 2003).

**Pepsin digestion:** The method (Fig 4) first involves the digestion of horse plasma proteins by pepsin, leading to a degradation of many non-IgG proteins, and to a cleavage of IgG into bivalent $F(ab')_2$ fragments by removal and digestion of the Fc fragment into small peptides. A heating step and the purification of $F(ab')_2$ fragments by salting-out using ammonium sulphate are also key elements of this methodology. Some procedures perform the peptic digestion step on a pre-purified IgG fraction that is obtained by ammonium sulphate treatment of plasma to obtain an IgG-enriched precipitate, whereas albumin is not precipitated. Pepsin digestion is accomplished at a

pH of 3.0-3.5. The incubation period ranges from 1 to 24 hours, at 30-37 °C in a jacketed tank. A typical protocol is based on incubation at pH 3.3 for one hour with a pepsin concentration of 1 g/L, although other procedures can be used with similar results. Each manufacturer should adjust the pepsin concentration in order to achieve the required enzymatic activity.

**Downstream processing using ammonium sulphate:** after pepsin digestion, the pH is adjusted to 4.5-5.0, by adding NaOH, to stop pepsin digestion; then, a solution of ammonium sulphate is added under stirring to a final concentration usually close to 12% (w:v). The precipitate is eliminated by filtration or centrifugation, and the filtrate, or supernatant, is heat-treated (usually at 56 °C for one hour; this is known as 'thermocoagulation'). The resulting fraction is filtered or centrifuged to eliminate the precipitate.The pH is then adjusted to 7.0-7.2 with NaOH, and a solution of ammonium sulphate is added under stirring to a final concentration high enough to precipitate the $F(ab')_2$ fragments (usually 23% (w:v) or higher). After an additional filtration step, or following centrifugation, the $F(ab')_2$ precipitate is dissolved, and then it is desalted (to eliminate the ammonium sulphate) and concentrated preferentially by tangential flow diafiltration. Care should be taken to avoid aggregate formation by ensuring gentle mixing and rapid dissolving of the precipitate. Alternatively, desalting can be achieved by dialysis initially against water for injection (WFI) and then against an isotonic saline solution. The $F(ab')_2$ solution is then formulated by adding NaCl, an antimicrobial agent and any other excipient needed for formulation, such as protein stabilizers, and the pH is adjusted, generally to neutral values. Finally, the preparation is sterilized by filtration on 0.22 µm filters, and dispensed into final containers (vials or ampoules). Such process, or similar ones developed by other manufacturers, using pepsin digestion, ammonium sulphate precipitation and tangential diafiltration is the most often used for the manufacture of $F(ab')_2$ fragments. The yield of this fractionation protocol usually ranges between 30 % and 40 %.

**Downstream processing using caprylic acid:** Purification of $F(ab')_2$ has also been shown, but only at an experimental scale, to be achievable by caprylic acid precipitation of non-$F(ab')_2$ proteins after pepsin digestion, with an improved yield (~60 %) (Raweerith and Ratanabanangkoon, 2003). However, the yield at large-scale is not known. Fig 5 shows a fractionation scheme of F(ab')2 using caprylic acid. $F(ab')_2$ are not precipitated, therefore reducing the formation of aggregates. Some manufacturers have introduced additional processing steps such as ion-exchange chromatography or ultrafiltration to eliminate low molecular mass contaminants (see below). Fig 4: Typical fractionation process of $F(ab')_2$ fragments by ammonium sulphate precipitation Fig 5: Typical fractionation process of $F(ab')_2$ fragment by caprylic acid precipitation

13.2.3 Purification of Fab antivenoms

Production of monovalent Fab fragments is performed by some manufacturers (Al-Abdulla et al., 2003), currently using sheep plasma. Papain is used in place of pepsin to the enzymatic digestion step, and the process of preparation of the fragment may use ammonium sulphate, sodium sulphate or caprylic acid. Fig 6 shows a process where immunoglobulins are precipitated from plasma by ammonium or sodium sulphate. The IgG precipitate is dissolved in a sodium chloride solution at pH 7.4. Papain is added and digestion performed at 37 °C for 18-20 hr in a jacketed tank.

Reaction is stopped by adding iodoacetamide. The product is then applied to a diafiltration system and equilibrated with a buffered isotonic sodium chloride solution. Afterwards, the preparation is chromatographed on an anion-exchanger (usually in QAE- or DEAE-based media). Fc fragments and other impurities are bound on the column, whereas Fab fragments pass through. After an additional diafiltration/dialysis step, the product is formulated by adding NaCl, antimicrobial agents (when used), any other excipients needed and the pH is adjusted. Finally, the preparation is sterilefiltered and dispensed into the final containers. Fig 6: Typical fractionation process of Fab fragments

13.2.4 Optional additional steps used by some manufacturers

When performed following GMPs and using validated fractionation protocols, the basic methodologies described above for the manufacture of IgG, $F(ab')_2$ and Fab antivenoms allow the production of antivenoms of adequate purity, safety and efficacy. Nevertheless, some manufacturers include additional steps to enhance product purity. The methodologies include: *13.2.4.1 Ion-exchange chromatography* Ion-exchange chromatography can be successfully used for antivenom purification based on charge differential with the contaminants. Anion-exchange columns of diethyl amino ethyl (DEAE) or quaternary ammonium (QAE) gels or membranes such as quaternary ammonium cellulose microporous membranes can be used at neutral pH to adsorb protein contaminants (Grandgeorge et al., 1996; Saetang et al., 1997; Jones and Landon, 2003). Alternatively, cationexchange columns, e.g. carboxymethyl (CM) or sulphopropyl (SP) gels, have been used for IgG or $F(ab')_2$ fragment purification (Raweerith and Ratanabanangkon, 2003). The column is equilibrated at acid pH, e.g. 4.5, to bind the antivenom, whereas proteins contaminants are eluted in the break-through. Chromatographic procedures should be applied following GMP. Columns should be adequately regenerated, sanitized, and stored in order to prolong their life-time. Measures to avoid batch to batch contamination should be in place. Specific SOPs should be developed and followed.

*13.2.4.2 Affinity chromatography*

Affinity chromatography using either immobilized venom or other ligands can be designed to bind immunoglobulins or their fragments (Sullivan and Russell, 1982). However, columns usually deteriorate rather rapidly, and meticulous care should be taken to wash, sanitize and store them under adequate conditions. Procedures should assure that any leaching substances from the columns do not affect the quality and safety of the product or are thoroughly eliminated during downstream processing; this is especially critical in affinity chromatography using immobilized venom. Affinity processes may impact recovery and high affinity antibodies may be lost and/or denatured due to the harsh elution conditions needed to elute them from the chromatographic material.

*13.2.4.3 Process improvement*

Some manufacturers have introduced process improvements to enhance the quality or the yield of antivenoms. Those include the use of: - Tangential flow filtration/ultrafiltration systems equipped with membranes of 30kDa to dialyze and concentrate the antivenoms - Depth filtration system combined with filter-aids to facilitate filtration steps and improve antivenom recovery

*13.2.4.4 Cost/benefit analysis*

A cost/benefit analysis is required when deciding the specific methodology to be used for antivenom production, especially considering the high cost of some fractionation steps. Moreover, considerations such as recovery of the product and ease of implementation of a fractionation protocol should be carefully considered. The introduction of chromatographic procedures significantly increases production costs and, therefore, their possible implementation should be carefully analyzed and justified.

13.2.5 Formulation

Formulation of antivenoms after diafiltration steps should consider the addition of salts to adjust the osmolality, preservatives, other excipients needed for protein stability, when required, and the adjustment of pH. In general, antivenoms are formulated at neutral pH although some manufacturers are exploring the feasibility of formulating at more acidic pHs to improve stability and/or to reduce aggregate formation. Formulation at pHs higher than 7.5 may not be recommended since the stability of immunoglobulins and their fragments at alkaline pH may be poor, and the formation of aggregates may be favoured.

13.2.6 Analysis of bulk product before dispensing

The biological, physical and chemical characteristics of the final bulk product should meet preestablished specifications before dispensing. Such analysis may include tests required to demonstrate the purity and potency of the product, the lack of aggregates, sterility and lack of pyrogens, and formulation in accordance with specifications. When the product is stored liquid, some of these tests (such as the potency assay) may not need to be duplicated on the final container, if the processing after the bulk preparation has been validated and shown not to alter this activity The sterilization equipment and the integrity of the membrane should be guaranteed before sterilization; moreover, the aseptic filling should be validated.

13.2.7 Dispensing and labelling of final product

Once compliance of the final bulk product with the quality control specifications is established, the final product is bottled. For this, final glass containers (vials or ampoules) of borosilicate quality should be used. General principles prevailing for the dispensing of parenteral medicinal products should be applied. The dispensing should be performed in class A clean room conditions, usually in a laminar flow hood. The equipment used for dispensing should be previously calibrated to assure that the correct volume is delivered. In the case of ampoules, the dispensing system should assure an aseptic closure and the sealing of the ampoule should avoid risk of protein denaturation due to heat. For vials, rubber toppers should be placed inside this clean dispensing area. The quality of rubber toppers should guarantee inertness and non-leaching. Thereafter, aluminum seals are placed on each vial in a clean area outside the class A area. Ampoules or vials containing the final product are then properly identified and stored in a quarantine area and maintained under proper storage conditions. Samples of the antivenoms are sent to the quality control laboratory for analysis. When an antivenom complies with all the quality control tests established for the final product, it should be properly labelled and identified. - The vial/ampoule should be labelled with, at least, the following information: name of the product and of the producer, animal species used to produce the antivenom, batch number,

pharmaceutical presentation (liquid or freeze-dried), volume content, administration route (intravenous/intramuscular), specificity (venoms that neutralize, including both the common and the scientific name of the snake(s)[1]), neutralising potency, storage conditions, and expiry date. Additional information may be requested by the national regulatory authorities. - The package, which is usually a cardboard box where the vials or ampoules are packed, should include the same information described on the primary container. - The package insert should include all the information related to the product, as established by national regulatory agencies, including the basic aspects of the correct therapeutic use of the antivenom, and warnings and contraindications.

13.2.8 Use of preservatives

The addition of preservatives, to prevent bacterial and fungal contamination, should be kept to a minimum during plasma storage and during fractionation. Its inclusion in the manufacture process should be clearly justified, and should never substitute any aspect of GMPs. Preservatives can be considered in the final product, especially in those manufactured in liquid presentation, and most specifically for multiple dose presentations. Antimicrobial agents currently used in antivenom formulation include phenol and metacresol. In general, phenol [1] Special care should be taken considering the frequent changes in snake species taxonomy concentration is adjusted to 2.5 g/L, and cresol concentration should be less than 3.5 g/L.The concentration of preservatives should be validated by each production laboratory on the basis of assays to test their efficacy. It is necessary to ascertain that any agent used does not have any potential detrimental interaction with the active substance and excipients of antivenoms. Any change in the formulation involving preservatives, or the elimination of preservatives from the final product, requires a very careful risk/benefit assessment on various microbial safety aspects, as well as detailed validation procedure.

13.2.9 Freeze-drying

Antivenoms are available either as liquid or freeze-dried preparations. Freeze-dried antivenoms, which may be stored at room temperature, are generally produced and distributed to markets where the cold chain cannot be guaranteed, such as occurs in many tropical regions of the world. The absence of guarantee of a cold chain during distribution highlights the need for manufacturers to demonstrate the stability of the antivenoms under the high temperatures found in tropical climates. Freeze-drying is a critical operation. Careful attention should be given to the freezing-rate as well as to the protocol used for the primary and secondary drying cycles (Pikal, 2002). The details of the freeze-drying protocols are product-specific and should be adjusted depending upon the particular formulation of each antivenom. Inadequate freeze-drying protocols may affect the physicochemical quality of the product, inducing protein precipitation and denaturation, as well as aggregate formation, and altering stability and reconstitution. Specific stabilizers, such as sugars or polyols, aimed at protecting proteins from denaturation and aggregation may be added to the final formulation of the antivenom.The use of bulking agents, frequently used for some biological products, is generally not required in the case of antivenoms owing to their relatively high protein concentration, however they are sometimes used for hightiter monospecific antivenoms.

13.2.10 Archive samples of antivenoms

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

### 13.3 Pharmacokinetic and pharmacodynamic properties of IgG, F(ab')2 and Fab

Owing to different molecular mass, the pharmacokinetics of heterologous IgG molecules and $F(ab')_2$ and Fab fragments differ significantly. In envenomed patients, Fab fragments have the largest volume of distribution and readily reach extravascular compartments. Fab fragments are, however, rapidly eliminated, mainly by renal excretion, thus having short elimination half-life (from 4 to 24 hours) (Meyer et al., 1997; Ariaratnam et al., 1999). In contrast, $F(ab')_2$ fragments and intact IgG molecules are not eliminated by the renal route and therefore have a more prolonged elimination half-life (between 2 and 4 days) (Ho et al. 1990; Scherrmann, 1994; Gutiérrez et al., 2003). Such different pharmacokinetic profiles have important pharmacodynamic implications, and the selection of the ideal type of active substance in an antivenom should rely on a careful analysis of the venom toxicokinetics and antivenom pharmacokinetics. Another difference between low molecular mass fragments, such as Fab on one hand, and higher molecular mass $F(ab')_2$ and IgG, on the other hand, is the number of paratopes of each molecule: Fab has one antigen binding site while IgG and $F(ab')_2$ each has two binding sites. Thus the latter will be able to form large and stable complexes/precipitates with antigens carrying several epitopes, while the former will form small, reversible non-precipitable complexes. Ideally, the volume of distribution of an antivenom should be as similar as possible to the volume of distribution of the main toxins in a particular venom; however, this is rarely the case. In venoms composed of low molecular mass toxins, such as some elapid snake venoms, low molecular mass neurotoxins are rapidly absorbed to the bloodstream and become rapidly distributed to the extravascular spaces where toxin targets are located. Furfthermore, low molecular mass toxins are eliminated from the body in a few hours. In these cases, an antivenom of high distribution volume that readily reaches extravascular spaces, such as as Fab, might be convenient, although their action is then eliminated within a few hours. It should be noted, however, that a number of elapid venoms contain some high molecular mass toxins of great clinical significance, such as procoagulants and pre-synaptic neurotoxins. In contrast, in the case of viperid snake venoms and other venoms made of toxins of larger molecular mass, including a number of elapid venoms, many of which act intravascularly to provoke bleeding and coagulopathy, the situation is different. The time required for toxins to distribute to extravascular spaces is longer than in the case of low molecular mass neurotoxins, and the targets of some of these toxins are present in the vascular compartment. In addition, the toxins of viperid venoms have a long half-life in vivo and can remain in the body for several days (Audebert et al., 1994; Choumet et al., 1996). In this case, an antivenom made by Fab fragments neutralizes the toxins that reach the circulation but, after a certain time lapse, the Fab fragments are eliminated and non-neutralized toxins reach the circulation, thus giving rise to the well-known phenomenon of recurrent envenoming, i.e., the reappearance of signs and symptoms of envenoming at later time intervals after an initial control of envenoming. This situation demands repeated administration of antivenom in order to maintain therapeutic levels of Fab in the circulation (Boyer et al.,

2001). Therefore, in such kinds of envenoming, antivenoms made of IgG or F(ab')2 may be more appropriate because of their prolonged elimination half-lives. Moreover, it has been proposed that formation of venom-antivenom complexes in the circulation results in the redistribution of venom components from the extravascular space to the blood compartment, where they are bound and neutralized by circulating antivenom, provided that the dose of antivenom is sufficient (Rivière et al, 1997, 1998). Consequently, the maintenance of a high concentration of specific antivenom antibodies in the circulation for many hours is required for complete neutralization of toxins reaching the bloodstream during both early and late phases of envenoming (redistribution of toxins) present in the extravascular space. In conclusion, IgG and F(ab')2 antivenoms have a pharmacokinetic profile that make them more effective in many types of snakebite envenoming.

### 13.4 Main recommendations

□ **Antivenoms should be manufactured using fractionation procedures that are well established, validated, and shown to yield products with proven safety and efficacy. Fractionation processes used for manufacture of antivenoms should adhere to the GMP principles of parenteral medicinal products.**

□ **Antivenoms can be composed of intact IgG molecule, F(ab')2 fragments or Fab fragments. Intact IgG antivenoms are preferentially produced by caprylic acid precipitation of non-IgG plasma proteins, leaving a highly purified IgG preparation in the supernatant or filtrate.**

□ **F(ab')2 fragment antivenoms are produced by pepsin digestion of plasma proteins, at acidic pH, usually followed by F(ab')2 purification by salting out with ammonium sulphate solutions or by caprylic acid precipitation. Fab monovalent fragments are obtained by papain digestion of IgG at neutral pH.**

□ **Further to ultrafiltration to remove low molecular mass contaminants, preparations are formulated, sterilized by filtration and dispensed in the final containers. Formulation of antivenoms may include preservative agents. Additional steps, such as chromatography, can be added to the fractionation protocols to enhance purity.**

□ **Antivenoms can be presented as liquid or freeze-dried preparations. Freeze-drying of antivenoms should be performed in conditions that ensure no denaturation of proteins and no formation of protein aggregates.**

□ **IgG, F(ab')2 and Fab antivenoms exhibit different pharmacokinetic profiles, Fab fragments having a larger distribution volume and a much shorter elimination halflife. Thus, for viperid envenomings, IgG or F(ab')2 antivenoms have a more suitable pharmacokinetic profile, whereas Fab fragments may be useful for the neutralization of venoms rich in low molecular mass neurotoxins of rapid distribution to tissues. However, in general terms, IgG and F(ab')2 antivenoms have shown a better pharmacokinetic profile than Fab antivenoms.**

## 14 CONTROL OF INFECTIOUS RISKS

### 14.1 Background

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

processing. There are currently several recognized complementary approaches used for virus risk reduction for biological products: - Minimising the potential initial virus content by implementing a quality system for the production of the raw material; - Contribution of the manufacturing processes at inactivating and/or removing residual viruses during manufacture of the biological product; such contribution can be inherent to the existing manufacturing technology or may result from the introduction of dedicated viral reduction steps - Adhesion to GMP at all steps of the production; - Appropriate and timely response to any infectious events recognized during the clinical use of the product. Manufacturing steps to inactivate and/or remove viruses have long been shown to play a powerful role in ensuring safety of biologicals (WHO, 2003) but keeping to a minimal the potential virus load at the stage of the plasma pool through appropriate epidemiological surveillance and animal health control is also an important safety aspect (see Section 10). Based on experience with human plasma products, it can be estimated that a production process of antivenoms that includes two robust steps of viral reduction (comprising preferably at least one viral inactivation step) should provide a satisfactory level of viral safety. However, it should be kept in mind that non-enveloped viruses are more difficult to inactivate or remove than lipidenveloped viruses.

**14.2 Risk of viral contamination of the starting plasma**

The main structural characteristics of viruses reported to possibly infect horses, sheeps and goats are presented in Tables 5 and 6. They include viruses with a DNA or RNA genome, with and without a lipid envelope, and ranging widely in size (22 to 300 nm). A few of these viruses have been identified as possibly present, at least at some stages of the infection cycle, in the blood or are considered as being pathogenic to humans. A specific attention should be paid to these viruses.

**Table 5: Viruses identified in horses (CPMP, 2002; Burnouf et al., 2004)**

**Virus Family Size (nm) Genome Presence in blood reported1 Classified as pathogenic to humans (CPMP, 2002) Enveloped** Equive Arteritis virus Arteriviridae 50-60 ss-RNA Borna virus Bornaviridae 70-130 ss-RNA Yes Yes Equine Encephalitis virus, Eastern & Western Togaviviridae 40-70 ss-RNA Yes Equine coronavirus Coronaviridae 75-160 ss-RNA Equine foamy virus Retroviridae 80-100 ss-RNA Yes Equine herpes virus1-5 Herpesviridae 125-150 Ds-DNA Yes Equine infectious anaemia virus Lentiviridae 80-100 ss-RNA Yes Equine influenza virus Orthomyxoviridae 80-120 ss-RNA Yes Equine morbilli virus (Hendra virus) Paramyxoviridae 150 ss-RNA Yes Japanese encephalitis virus Flaviviridae 40-70 ss-RNA Yes Nipah virus Paramyxoviridae 150-300 ss-RNA Yes Rabies virus Rhabdoviridae 75-180 ss-RNA Yes Salem virus Paramyxoviridae 150-300 ss-RNA St-Louis encephalitis virus Flaviviridae 40-70 ss-RNA Yes Venezuelan equine encephalitis virus Togaviridae 40-70 ss-RNA Yes Yes Vesicular stomatitis virus Rhabdoviridae 50-80 ss-RNA Yes Yes West Nile virus Flaviviridae 40-70 ss-RNA Yes Yes **Non-enveloped** Equine encephalosis Reoviridae 80 Ds-RNA Equine rhinitis A and B viruses Picornaviridae 22-30 ss-RNA Equine rotavirus Reoviridae 60-80 Ds-RNA 1 Absence of report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection

**Table 6: Viruses identified in sheep and goat (CPMP, 2002)**

**Virus Family Size (nm) Genome Reported presence in blood 1 Classified as Pathogenic to Humans (CPMP, 2002) Enveloped** Wesselbron virus Flaviviridae 40-50 ss-RNA Yes Loiping ill virus Flaviviridae 40-50 ss-RNA Yes Rift valley fever complex Bunyaviridae 80-120 ss-RNA Yes Tick-borne encephalitis virus Flaviviridae 40-50 ss-RNA Yes Bluetongue virus Reoviridae 80 ds-RNA Yes Vesicular stomatitis virus Rhabdoviridae 50-380 ss-RNA Yes Yes Poxviruses (Parapox, Capripox, Cowpox) Poxviridae 140-260 ds-DNA Yes Parainfluenza virus type 3 Paramyxoviridae 150-300 ss-RNA Yes Borna virus Bornaviridae 70-130 ss-RNA Yes Yes Respiratory syncytial virus Paramyxoviridae 150-300 ss-RNA Rotavirus Reoviridae 80 ds-RNA Akabane virus Bunyaviridae 80-120 ss-RNA Ovine herpes virus 2 Herpesviridae 120-200 ds-DNA Bovine herpes virus types 1, 2, 4 Herpesviridae 120-200 ds-DNA Border disease virus

Flaviviridae 40-70 ss-RNA Ovine/bovine papillomavirus Papillomaviridae 40-55 ds-DNA Bovine viral diarrhoea virus Togaviridae 40-60 ss-RNA Retroviruses (Caprine arthritis encephalitis virus, Maedi-Visna virus, virus, Jaagsiekte virus, bovine leukaemia virus Retroviridae 80-100 ss-RNA Peste des petits ruminants (Morbillivirus) Paramyxoviridae 150-300 ss-RNA Adenovirus Adenoviridae 80-110 ds-DNA Nairobi sheep disease Bunyaviridae 80-120 ss-RNA Ross river virus Togaviridae 70 ss-RNA **Non-enveloped** Foot and mouth disease virus Picornaviridae 27-30 ss-RNA Yes Reovirus 1-3 Reoviridae 60-80 ds-RNA Epizootic haemorrhagic disease Virus Reoviridae 80 ds-RNA 1 Absence of report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection

### 14.3 Viral validation of manufacturing processes

Understanding how much a manufacturing process may contribute to the viral safety of antivenoms is fundamental to both manufacturers and regulators. Such an understanding can only be achieved by viral validation studies. These studies are complex and require wellestablished virology laboratory infrastructure and cell culture methodologies. They are usually carried out by specialized laboratories, outside of the manufacturing facilities. The principles guiding such studies have been described in WHO Guidelines (WHO, 2003) The principles to be used for the validation of manufacturing processes of antivenoms are summarized briefly here, and it is therefore recommended to refer to these Guidelines for full details.

14.3.1 Down-scale experiments

The contribution of manufacturing processes to inactivate and/or remove potential viral contamination should be demonstrated. For that purpose, viral validation studies should be performed using at least three viruses exhibiting different structural characteristics. The antivenom manufacturer should first identify the steps that, based on existing literature, are likely to remove or inactivate viruses and, then, provide evidence and quantitative assessment of the extent of virus reduction achieved for the specific process evaluated. Validation should be done by down-scale experiments. The accuracy of the down-scale process should be assessed by comparing the characteristics of the starting intermediate and the fraction resulting from that step, for both the laboratory and the manufacturing scales. Selected physical factors (e.g., temperature, stirring, filtration conditions, etc.) and chemical factors (e.g., pH and the concentration of precipitating agents such as caprylic acid, etc.) should be equivalent where possible to those used at manufacturing scale. Once the step is accurately modeled, the antivenom fraction derived from the fractionation process just prior to the step being evaluated (e.g. the starting plasma to be subjected to a low pH treatment, or to caprylic acid precipitation, or a $F(ab')_2$ fragment fraction to be subjected to an ammonium sulphate-heat treatment) should be spiked with one of the model viruses selected. Viral infectivity, most often determined using cell culture assays (less frequently animal models), should be quantified prior to (e.g. prior to pH adjustment and addition of pepsin) and right after (e.g. after low pH adjustment and incubation at that pH for a known period of time in the presence of pepsin) the steps evaluated to determine the viral clearance achieved. The results are conventionally expressed in terms of the logarithm (log) of the reduction in infectivity that is observed. Total infectivity or viral load is calculated as the infectious titer (infectious units per ml) multiplied by the volume. For a viral inactivation step, it is highly recommended that the kinetics of the virus kill be evaluated. Such inactivation kinetics of the infectivity indeed provides an important indication of the virucidal potential of the step and enables to compare data to those from published studies. Typically, an extent of 4 logs or more

viral reduction is considered to represent an effective and reliable viral safety step. Establishing the relative insensitivity of a manufacturing step to changes or deviatons in process conditions is also important to evaluate its robustness, in addition to allowing building-up the level of understanding of its contribution to the overall viral safety of the preparation. This can be achieved by validating the same step using a range of conditions deviating from those used in production (such as a upper pH limit applied to a pepsin digestion or to a caprylic precipitation step). Virus validation studies are subject to a number of limitations (WHO, 2003) which should be considered when interpreting the results.

14.3.2 Selection of viruses for the validation of antivenoms production processes Viruses selected for viral validation studies should resemble as closely as possible those which may be present in the starting animal plasma material (Tables 5 and 6). When possible, viruses known to potentially contaminate animal plasma (they are called "relevant viruses") should be used. Table 7 gives examples of a few viruses that have been used for the validation of animal derived antisera. Vesicular stomatitis virus (VSV) and West Nile virus (WNV) are relevant lipidenveloped horse plasma-borne viruses. Bovine viral diarrhea virus (BVDV), a lipid-enveloped flavivirus, can be used as a model for West Nile virus (WNV) and for the Eastern, Western, and Venezuelan Equine Encephalitis togaviruses. Pseudorabies virus (PRV) is a lipid-enveloped virus that can serve as a model for pathogenic equine herpesvirus. Finally, encephalomyocarditis virus (EMCV), a picornavirus, can serve as a model for non-lipid-enveloped viruses. This list is not limitative and other model viruses can be used for validation studies of animalderived antivenoms, in particular taking into account the characteristics of the viruses that may be present in the animal species used to generate antivenoms.

**Table 7: Examples of laboratory model viruses that can be used for validation studies of horse-derived antivenoms** **Virus Family Envelope Size (nm) Genome Resistance Model for** Vesicular stomatitis virus Rhabdoviridae Yes 50-200 ss-RNA Low Relevant virus West Nile virus Flaviridae Yes 40-70 ss-RNA Low Relevant virus and model for Eastern equine encephalitis Virus Sindbis virus Togaviridae Yes 60-70 ss-RNA Low Eastern, Western and Venezuelan equine encephalitis virus Bovine virus Diarrhoea virus Togaviridae Yes 40-60 ss-RNA Low Eastern, Western and Venezuelan equine encephalitis virus Parainfluenza virus Paramyxoviridae Yes 100-200 ss-RNA Low Hendra virus; Nipah virus; Salem virus Pseudorabies virus Herpes Yes 100-200 ds-DNA Medium Equine herpes virus Reovirus type 3 Reoviridae No 60-80 ds-RNA Medium Equine encephalosis virus Poliovirus; Encephalomyocarditis virus; Hepatitis A virus Picornaviridae No 25-30 ss-RNA Mediumhigh Equine rotavirus

**14.4 Viral validation studies of antivenoms or other animal-derived antisera** There is no documented case of transmission of zoonotic infections, including viral diseases, by animal derived antivenom immunoglobulins, or any other animal-derived antisera. Absence of viral transmissions may result from a lack of long-term surveillance of the patients receiving antivenoms. Alternatively, this may also reveal that current manufacturing processes of antivenoms include processing steps that contribute to the viral safety. Among the various processing steps used in the manufacture of antivenoms, caprylic acid and low pH treatments are known to contribute to safety against lipid-enveloped viruses. This information is based on well-established experience in the fractionation of human plasma with manufacturing step comprising caprylic acid (Dichtelmuller et al., 2002; Korneyeva et al., 2002; Parkinnen et al., 2006)

or low pH treatment (Reid et al, 1988; Omar et al, 1996; Bos et al., 1998; WHO, 2003). Although information is still limited, there is now building evidence that similar steps used in the production of antisera, and more specifically antivenoms, may also inactivate or remove viruses. In addition, some manufacturers have implemented dedicated viral reduction procedures.

14.4.1 Caprylic acid treatment

The conditions used for caprylic acid treatment of antivenoms (Burnouf et al., 2004) and of human immunoglobulins (Dichtelmuller et al., 2002; Korneyeva et al., 2002; Parkinnen et al., 2006), are similar, in particular the pH range, duration of treatment, temperature, and the caprylic acid/protein ratio, as summarized in Table 8.

**Table 8- Comparison of conditions for caprylic acid treatment used for human immunoglobulins preparations and antivenoms (Burnouf et al., 2004)** Product Protein concentration (g/l) Caprylate/kg solution (g) pH Temperature (°C) Duration (hr) Human IgG 35 7.45 5.5 22 1 Human IgMenriched 43 15 4.8 20 1 Human IgM 25 20 5.0 20 1 Antivenoms 60 to 90 50 5.5 to 5.8 18 to 22 1 *14.4.1.1 Validation studies with human immunoglobulins* Unsaturated fatty acids, most specifically caprylic acid, have long been known to have the capacity to inactivate enveloped viruses in human plasma protein fractions (Horowitz et al., 1998; Lundblad and Seng, 1991). The non-ionised form of caprylic acid is thought to disrupt the lipid bilayer and membrane associated proteins of enveloped viruses. Utilizing the dissociation reaction and varying the concentration of the ionized form of caprylate, a specific amount of the nonionized form of caprylate can be maintained over a wide pH range. The robustness of a caprylic acid treatment applied to human immunoglobulin G and M (IgM) preparations (IgG; IgM-enriched; and IgM preparations) has been investigated using various enveloped viruses (Human immunodeficiency virus –HIV-, BVDV, Sindbis Virus and Pseudorabies) (Dichtelmuller et al., 2002). Complete inactivation (_4.68 to _6.25 logs) was found within the first minutes. Viral inactivation in this human Ig products was not affected by pH (5.5 – 6.0), temperature (0-26°C), and protein content (30-40 g/l). Above pH 6, and most specifically at pH 8, no BVDV inactivation was found. At a content of caprylic acid of 3.7 g/kg or less, inactivation of HIV is significantly reduced. Under the conditions applied during manufacture, caprylic acid leads to robust inactivation of lipid-enveloped viruses; pH is a particularly critical parameter and should be less than 6. These conditions have also been found to inactivate >4.7 log10 of Equine arteritis virus (EAV), an equine virus used a model in these studies (Burnouf et al. 2004). Another study studied the viral reduction achieved during caprylic acid precipitation of non-IgG proteins from human IgG product (Korneyeva et al., 2002). At pH 5.1, 23°C, and in the presence of 9 mM caprylate, _ 4.7 and _ 4.2 log of HIV and PRV, respectively, were inactivated during the 1hr treatment, but only 1.5 log for BVDV was inactivated. At 12 mM caprylate, _4.4 log of BVDV were inactivated within this time period. At pH5.1, 24°C, 19 mM caprylate, and pH 5.1, 24°C, 12 mM caprylate, complete inactivation of BVDV and of HIV and PRV was achieved in less than 3 min.

*14.4.1.2 Validation studies with antivenoms*

Virus inactivation studies have been carried out on an F(ab')2 obtained from a pepsin digested plasma subjected to ammonium sulphate-precipitation. The F(ab')2 fraction was subjected to precipitation by drop-wise addition of caprylic acid to 0.5% (final

concentration) and the mixture was maintained under vigorous stirring for 1 hour at 18 °C. Rapid and complete reduction of BVDV, PRV, and VSV (>6.6 $log_{10}$, >6.6 $log_{10}$, and >7.0 $log_{10}$, respectively) was found. As expected, no significant reduction (0.7 $log_{10}$) of the non-enveloped EMCV (Burnouf et al, 2007) was observed. In another process, used to prepare equine immunoglobulins, where serum is thawed at 4°C, subjected to a heating at 56°C for 90 min, brought to 20 +/- 5 °C, adjusted to pH 5.5 and subjected to 5% caprylic acid treatment for 1 hour, fast reduction of infectivity of > 4.32 and > 4.65 $log_{10}$ was found for PRV and BVDV, respectively. The caprylic acid step was confirmed to have limited impact on the infectivity EMCV and MVM non-enveloped viruses (Mpandi et al, 2007). Data suggest that significant reduction in the infectivity of lipid-enveloped viruses can be obtained during caprylic acid treatment of antivenoms. The reduction of viral infectivity may result from both viral inactivation and partitioning during the precipitation step. No significant inactivation of non-enveloped viruses is expected.

*14.4.1.3 Recommended actions*

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

*14.4.1.4 Acid pH treatment:* The conditions used for low pH treatment of equine antivenoms and of human immunoglobulins are summarized in Table 9.

**Table 9: Typical conditions for acid pH treatment of human IgG preparations and equine antivenoms (Burnouf et al., 2004)** Product Protein concentration (g/l) pH Temperature (°C) Duration (hrs) Human IgG 40 to 60 4 30 to 37 20 to 30 Antivenoms 60 to 90 3.1 to 3.3 30 to 37 0.6 to 24

*14.4.1.5 Validation studies on human IgG*

Many studies have demonstrated that the low pH 4 treatment used in the manufacture of human intravenous IgG has the capacity to inactivate enveloped viruses (Reid et al, 1988; Omar et al, 1996; Bos et al., 1998). The rate and extent of inactivation may defer depending upon the viruses. Pepsin is sometimes added in traces (to reduce anticomplementary activity and content of aggregates) but, at this low concentration, contributes little to virus kill (WHO, 2003). Inactivation of viruses is temperature-dependent, and the reaction rate is influenced by the solute composition of the IgG solution (Omar et al., 1996). Inactivation of some non-enveloped viruses has been found in some pH 4 treatments, but the rate and extent of inactivation is generally less than that for enveloped viruses (Bos et al. 1998). Some non-enveloped viruses are very resistant (Reid et al., 1988),

*14.4.1.6 Validation studies with antivenoms*

As described in Chapter 13, peptic cleavage of horse plasma IgG at pH 3.0 to 3.3 for 60 min is a common procedure for the preparation of $F(ab')_2$. More than 4 logs of inactivation of WNV and of Sindbis has been found when horse plasma was subjected to peptic digestion at pH 3.2 for 60 min (Lazar et al., 2002). WNV was very sensitive pepsin was added or not, whereas Sindbis inactivation rate and extent was higher in the presence of pepsin. This indicates that pH 3.2 alone inactivates WNV, while other phenomena involving the action of pepsin contributes to Sindbis inactivation at low pH. Confirmation of the significant inactivation of lipid-enveloped viruses during peptic

cleavage of plasma at pH 3.2 was found by another group (Burnouf et al, 2007). In this process, plasma is diluted with two volumes of saline, pH adjusted to 3.3, and pepsin is added to a final concentration of 1g/L. The mixture is incubated at pH 3.3 for 1 hour. >5.1 $log_{10}$ inactivation of PRV occurred is less than 6 minutes and > 7.0 $log_{10}$ in 60 min. There was >3.1 $log_{10}$ and >4.5 $log_{10}$ inactivation of VSV after 6 and 20 min, respectively. The reduction of infectivity of BVDV was less: 1.7 $log_{10}$ after 60 min. Inactivation of EMC, a non-enveloped virus, was relatively slow but reached between 2.5 and 5.7 $log_{10}$ after 60 min of pepsin incubation. This showed that reduction of infectivity of at least some non-enveloped viruses may take place during peptic digestion of diluted horse plasma. This does not mean, however, that other non-enveloped viruses would be inactivated to the same extent under such conditions.

*14.4.1.7 Recommended actions*

Manufacturers of F(ab')2 antivenoms are encouraged to validate the pepsin digestion process since virus inactivation is likely to be influenced by pH, time, temperature, pepsin content, and protein content. Studies of process robustness to define the impact on process variations are also recommended *14.4.1.8 Filtration steps* Other steps used in antivenom manufacture may contribute to viral safety through unspecific viral removal. The virus removal capacity of two depth-filtration steps performed in the presence of filter aids and used in the manufacture of equine antisera produced by ammonium sulphate precipitation of pepsin-digested IgG has been evaluated (Cameron-Smith et al., 2000). Clearance factors of 5.7, and 4.0 $log_{10}$ have been found for two enveloped viruses (infectious bovine rhinotraceheitis virus and canine distemper virus, respectively) and of 5.3 and 4.2 $log_{10}$ for two non-enveloped viruses (canine adenovirus virus and poliovirus type I, respectively). However, it should be kept in mind that viral reduction obtained by non-dedicated removal steps are usually regarded as less robust that dedicated viral inactivation or removal steps (WHO, 2003).

14.4.2 Validation of dedicated viral reduction treatments

*14.4.2.1 Pasteurisation and other liquid heat-treatments*

Pasteurisation is defined as the treatment of a liquid protein fraction for 10 hours usually at 60°C. It is a well-established viral inactivation treatment of human plasma products, such as immunoglobulins G (WHO, 2003). It is being used in the production process of a few equinederived immunoglobulins (Grandgeorge et al., 1996). Validation studies showed that heating a purified equine immunoglobulin at 58°C+/-0.1°C without stabilizers inactivates _4.8 $log_{10}$ of PRV, _4.3 $log_{10}$ of BVDV in less than 30 min, and > 4.7 log of EMCV in less than 1 hour. In contrast, infectivity of Minute Virus of Mice (MVM), a non-enveloped virus, was still detected after 9 h and 30 min of treatment; only 1.59 $log_{10}$ were inactivated (Mpandi et al, 2007).

*14.4.2.2 Nanofiltration*

Nanofiltration is a technique of filtration specifically designed to remove viruses, based on size while permitting flow-through of the desired protein (Burnouf et al., 2005). Effective viral removal requires, in principle, that the pore size of the filter be smaller than the effective diameter of the virus particles. Robust removal of both lipid-enveloped and non-enveloped viruses has been reported during 15nm filtration of an Fab antivenom against North American viperid snake venoms. Nanofiltration of intact IgG is expected to be more difficult to achieve on the current small poresize

membrane.

14.4.3 Other viral inactivation treatments currently not used in antivenoms manufacture

Other viral inactivation methods have been developed to ensure the viral safety of biological products. Those include, in particular, a treatment with a combination of an organic solvent (trin- butyl phosphate or TnBP) at concentrations between 0.3 and 1%, and detergents such as Triton X-100 or Tween 80, also at concentrations generally comprised between 0.3% and 1%. Such solvent-detergent (S/D) procedures have been proven to be very efficient and robust in the inactivation of lipid-enveloped viruses in human plasma products (WHO, 2003). However, its use has not been reported for antivenoms. Implementation of dedicated viral inactivation treatments, such as S/D, should be encouraged for processes which, based on risk assessment, would offer insufficient margin of viral safety. Process changes associated with the introduction of new viral reduction steps, and the subsequent removal of any toxic compounds needed for viral inactivation, should be demonstrated not to affect the quality and stability of antivenoms, and most particularly the neutralization efficacy of venoms. Preclinical assessment of the possible impact of newly introduced viral inactivation treatments should be mandatory.

14.4.4 Possible contribution of phenol and cresols

The anti-bacterial agents, phenol or cresol (and more rarely formaldehyde) are added, by most manufacturers to the starting plasma donations as well as to the final liquid antivenom preparations, at a maximum final concentration of 0.25 to 0.35 %. Compounds such like phenol are known to be very lipid-soluble and lipophilic. Phenol used at a final concentration higher than 0.1 %, a temperature above 2°C, and for a duration of 30 to 60 min, has been shown to have, within 30 to 60 min, a significant virucidal effect in human blood products (Louderback, 1992a,b). Performing validations of the potential virucidal effect of antimicrobial agents as added to the starting hyperimmune plasma and to the final antivenom preparations is highly encouraged. More information is urgently needed on the impact of this antimicrobial agent on the viral safety of antivenoms. This should be carefully weighed taking into consideration the potential benefits that these agents may have on the viral safety.

**14.5 Production-scale implementation of process steps contributing to viral safety**

As there is increasing, although preliminary, evidence that at least some existing manufacturing steps of antivenoms contribute to viral reduction, it is already recommended that specific care should be developed to ensure their appropriate industrial implementation in order not to compromise any possible benefits they provide in viral safety. Measures should therefore be taken to ensure and assess that such steps are correctly carried out in a manufacturing environment and that cross- and downstream- contamination are avoided. Such important aspects in product safety have been highlighted recently in WHO Guidelines (WHO, 2003) and should also be taken into consideration for large-scale operations of antivenoms. Specific attention should be given to:

_ Process design and layout, in particular the production floor area needed to implement safely such treatment, the justification to create a safety zone avoiding risk of downstream contamination, and the procedures used for cleaning and sanitization of the equipment to avoid batch to batch cross-contamination

_ Equipment specifications, having in mind the contribution for viral reduction. For instance, vessels used for low pH incubation or caprylic acid treatment should be fully enclosed and temperature-controlled. There should be no "dead points" where the temperature defined in the specification or where mixing homogeneity cannot be ensured. A poor equipment design has the potential to compromise the viral safety theoretically afforded by a given production step.
_ Qualification and validation should verify that the equipment conforms to predefined technical specifications and relevant GMP.
_ Process implementation: Production steps contributing to viral safety such as low pH treatment and caprylic acid treatments could be implemented in two stages. The first stage may be located in a normal production room, followed by a second incubation in another tank located in a segregated, contained area.
_ Process control: It is a critical part of the manufacturing process since completion of viral inactivation and removal cannot be guaranteed by testing final product. Samples should be taken to confirm that the process conditions of claimed inactivation steps meet the specified limits (e.g., pH, stabilizers concentration, concentration of virus inactivating agent, such as caprylate). When this is technically feasible and intermediates are stable, samples can be kept frozen for possible additional analysis prior to the release of the batch. It is Quality Assurance's (QA) responsibility to ensure that the execution of steps contributing to virus inactivation and removal in a production setting conforms to the conditions that contribute to such virus reduction.
_ Standard Operating Procedures: steps contributing to viral reduction should be described in approved Standard Operating Procedures (SOPs). Those SOPs should contain critical process limits for the viral inactivation and removal methods.
_ Role of the Quality Assurance department: because of the critical nature of the viral inactivation and removal step, QA personnel should review and approve the recorded conditions for viral inactivation and removal while the batch is being processed; i.e., not just as part of the final overall review of the batch file.

**14.6** Transmissible spongiform encephalopathy (TSE)

There has been no cases of transmission of transmissible spongiform encephalopathy (TSE) linked to medicinal product TSE has not been identified in the equine species. There has been no case of transmission of TSE linked to antivenoms or other equine-derived blood products. Of particular concern, however, is the fact that TSEs include scrapie in sheep, a ruminant species that is used, although much less frequently than horse, in the manufacture of antivenoms. Scrapie is a disease similar to bovine spongiform encephalopathy (BSE or "mad-cow" disease), but is not known to infect humans. However, the blood of sheep with experimental BSE or natural scrapie can be infectious and, because scrapie and BSE prion agents behave similarly in sheep and goats, the blood of small ruminants should either be avoided in preparing biologicals or selected very carefully from sources known to be free of TSEs. The recent findings of diseaseassociated proteins in muscles of sheep with scrapie and the recognition of BSE itself in a goat, reinforce the need for manufacturers of biologicals, including antivenoms, to maintain the precautionary safety measures recommended in the WHO guidelines on TSEs Tissue Infectivity (WHO TSE, 2006). According to these recommendations, tissues or body fluids of ruminant origin should be avoided in the

preparation of biological and pharmaceutical products. When sheep materials must be used, they should therefore be obtained from sources assessed to have negligible risk from the infectious agent of scrapie. Documented surveillance should be available. The infectious agent is thought to be a misfolded, abnormal, prion protein. $PrP_{TSE}$. At the present time, it is not known whether manufacturing processes used to produce antivenoms from sheep plasma include steps that can contribute to the removal of $PrP_{TSE}$. Experimental prion clearance studies, based on spiking experiments, can be performed to assess the capacity of the process to remove prions. However, there is still uncertainty on the validity of such experimental studies since the biochemical features of $PrP_{TSE}$ in blood and plasma is not known.

**14.7 Main recommendations**

□ **The viral safety of antivenoms results from a combination of measures to ensure satisfactory health status of the animals, reduced risk of contamination in the starting raw material, contribution of the manufacturing process to inactivate and/or remove viruses, and compliance to good manufacturing practices all along the chain of production.**

□ **Manufacturing processes should include at least one, preferably two steps contributing to robust viral reduction. A step of virus inactivation that can be easily monitored is usually preferred to other means of viral reduction, such as unspecific removal.**

□ **Manufacturers are encouraged to evaluate and validate the capacity of their current manufacturing processes (in particular low pH pepsin digestion, caprylic acid treatment, ammomium sulphate/heat precipitation, and possibly other steps) to inactivate or remove viruses. These studies should be done following existing international Guidelines and using relevant and/or model viruses that are representative of the viruses that could affect the animal used for raising the antivenoms immunoglobulins.**

□ **Considerations to remove antimicrobial agents from the final formulation of antivenoms should be carefully weighed taking into consideration the potential benefits these agents may have on the viral safety.**

□ **Should the viral reduction found for processes used be found insufficient to ensure a margin of safety, the introduction of dedicated viral reduction methods should be considered. The impact of such process changes on product efficacy and safety should be carefully analyzed in vitro as well as in preclinical studies prior to performing human clinical evaluations.**

□ **Great attention should be given to the production-scale implementation of all steps contributing to viral safety to ensure a consistent and reproducible batch-to-batch viral reduction and an absence of risks of cross and downstream re-contaminations that would jeopardize the viral safety of the product**

□ **When sheep materials must be used for the production of plasma, they should be obtained from sources assessed to have negligible risk from the infectious agent of scrapie.**

## 15 QUALITY CONTROL OF ANTIVENOMS

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

approved for each antivenom product or the respective intermediates, and are part of the batch record. For a liquid preparation, some quality control tests, such as the potency test or the detection of residual reagents used during fractionation, can be performed on the final bulk and may not need to be repeated on the final product, if the processing after the bulk preparation has been validated not to have an impact. The quality control of the final product in antivenoms includes the following tests;

**15.1 Routine assays**

15.1.1 Solubility (freeze-dried preparations)

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

15.1.2 Extractable volume:

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

15.1.3 Venom-neutralising potency test

This test determines the effectiveness of the antivenom to neutralise the overall toxic activity (the combined effects of the venom components responsible for) of the snake venom(s) against which the antivenom is designed. The first section of the test, to determine the lethal activity of the venom, is called the Median Lethal Dose ($LD_{50}$) assay and usually utilises mice of 18-20 g weight range. For new venoms whose $LD_{50}$ is unknown, it is recommended that a range dosefinding study, using one mouse per venom dose, is performed to avoid using excessive numbers of animals. LD(50) Range-Finding Test: Various venom doses are prepared using saline solution as diluent, and aliquots of 0.2 ml of each dose are injected, using one mouse per dose, by the intravenous route in the tail vein (or, alternatively, by the intraperitoneal route (using injection volumes of 0.5 ml). Deaths are recorded at 24 hours (intravenous test) or at 48 hours (intraperitoneal test). On the basis of this preliminary dose-finding experiment, a range of venom doses causing 0% to 100% lethality is established and thus narrows the range of venom doses required to formally estimate the toxic activity of the venom. The Median Lethal Dose ($LD_{50}$) assay: Groups of 5-6 mice of a defined weight range are injected intravenously, in the tail vein, with 0.2 mL of solutions of varying doses of venom dissolved in saline solution. A minimum of 5 mice is the smallest number recommended for obtaining statistical significance. In some laboratories the $LD_{50}$ is estimated by the intraperitoneal route using an injection volume of 0.5 mL. Deaths are recorded at 24 hr (for assays involving intravenous injections) or at 48 hr (intraperitoneal injections), and $LD_{50}$ is estimated by Probit analysis (Finney, 1971), Spearman-Karber (WHO, 1981) or alternative procedures (such as non-parametic methods). One venom $LD_{50}$ is defined as the minimal amount of venom causing death in 50% of the mice injected. The test to assess the neutralizing potency of an antivenom is called the Median Effective Dose ($ED_{50}$) assay. For a new antivenom, it is recommended that a preliminary range dose-finding procedure is performed, using one mouse per antivenom dose. ED(50) Range-Finding Test: The selected multiple of the venom $LD_{50}$ (3-5 $LD_{50}$) is mixed with different doses of antivenom and incubated at 37°C for 30 minutes and each mixture injected into a single mouse. This preliminary

test should establish a range of antivenom volumes that result in 100% survival and 100% death of the injected mice and thus narrows the range of doses required for the formal ED(50) test. The Median Effective Dose ($ED_{50}$) assay: This test involves the incubation of a fixed amount of venom ('challenge dose', usually corresponding to three to five $LD_{50}$s), with various volumes of the antivenom adjusted to a constant final volume with saline solution (WHO, 1990; Theakston et al., 2003; Rojas et al., 2005). The mixtures are incubated for 30 minutes at 37 °C, and then aliquots of 0.2 mL of each mixture are injected into groups of 5-6 mice of a defined weight range by the intravenous route, using the tail vein. A control group injected with a mixture of the venom 'challenge dose' with saline solution alone (no antivenom) should be included to confirm that the venom 'challenge dose' induces 100% lethality. When the test is performed by the intraperitoneal route, a volume of 0.5 mL is administered. Centrifugation of the antivenom-venom mixtures is not recommended because residual venom toxicity may remain in the immunoprecipitates. After injection, deaths are recorded at 24 hours (intravenous injection) or at 48 hours (intraperitoneal injections) and the results analysed using Probit analysis (Finney, 1971), Spearman-Karber (WHO, 1981) or alternative procedures (such as non-parametic methods). The Median Effective Dose ($ED_{50}$) of an antivenom is defined as the volume of antivenom that protects 50% of the mice injected. The $ED_{50}$ can be expressed in various ways:

(a) mg of venom neutralized by mL of antivenom;
(b) µL antivenom required to neutralize the 'challenge dose' of venom used;
(c) µL of antivenom required to neutralize one mg of venom; and
(d) number of $LD_{50}$s of venom neutralized per mL of antivenom.

Every production laboratory and every national regulatory agency should establish the accepted levels of neutralizing potency for the various antivenoms being produced and distributed. In this regard, it is important to guarantee that a standardized assay is used by the manufacturing laboratories. Since the methodology to estimate antivenom potency (ie, $ED_{50}$) varies between laboratories and countries, manufacturers should disclose the conditions in which the potency of their antivenoms is estimated to the correspondent regulatory agencies in the course of their licensing and control procedures. The protocols for the selection and quality control of the venoms used for these potency assays should be established in each quality control laboratory (see Section 8). Venoms used in this test should correspond to a representative pool obtained from at least 25-50 well-identified snake specimens collected from various regions within the geographical range of distribution of the species in a country. These national reference venom pools must be evaluated periodically in order to assure that they have not deteriorated (see Section 8 on quality control of venoms). Until *in vitro* or alternate tests of lesser severity become accepted, these venom LD(50) and antivenom ED(50) assays should be performed by all manufacturers before an antivenom can be used in humans. The assays should be conducted under conditions causing the minimal possible suffering to the experimental animals.

15.1.4 Osmolality

Osmolality can be measured to determine the tonicity of the antivenoms. It is recommended that it be more than 240 mosmom/kg. Determination of osmolality is also an indirect means to determine the quantity of salts or excipients added for formulating

the batch.

15.1.5 Identity test

When several types of antivenoms are produced by a single laboratory, the identity of each batch of antivenom should be controlled. Identity tests may include biological assays as well as physico-chemical and immunological tests. Double immunodiffusion assays, confronting the antivenom with the venoms against which the antivenom is designed, are often used. In the case of laboratories that use various animal species to raise antivenoms, i.e. horses and sheep, an immunological identity test should be used to identity the mammalian species in which the antivenoms are produced. The potency assay against venoms is another way to identify antivenoms.

15.1.6 Protein concentration

The total protein concentration of antivenoms is performed using the Kjeldahl method for nitrogen determination. Alternatively, several colorimetric procedures can be used, measuring absorbance at 280 nm. The presence of preservatives should be taken into account since they may interfere with some protein determination methods (Tuck and Ng, 2005). The total concentration of proteins in antivenoms should not exceed 10 g/dL, unless a higher protein content is justified and authorised by the competent authority.

15.1.7 Purity

The purity of the active substance, i.e. intact immunoglobulin or immunoglobulin fragments, should be assessed., They should constitute the great majority of the preparation, ideally greater than 90%. Electrophoretic methods in polyacrylamide gels (SDS-PAGE run under reducing or nonreducing conditions) are suitable for this purpose, since these techniques allow the detection and monitoring of IgG, $F(ab')_2$, Fab, non-IgG plasma protein contaminants (in particular albumin), and degradation products. The electrophoretic pattern should be compared to that of a reference preparation. A semi-quantification can be performed by calibration of the procedure. Of particular relevance is the assessment of the albumin content which ideally should not exceed 1 % ot total protein content. The following approach can serve as a guide in assessing the purity of antivenoms:

**SDS-PAGE under non-reducing conditions.** This analysis can inform qualitatively (or, at best, semi-quantitatively) on the amounts of intact immunoglobulins, digestion products and, importantly, on the presence of high molecular weight oligomers (soluble aggregates) and low molecular mass contaminants (which are expected in the case of enzymatically-digested antivenoms).

**SDS-PAGE under reducing conditions.** Analysis under these conditions can inform on the amount of immunoglobulins and their fragments by direct visualization of intact and/or digested immunoglobulin heavy chains.

15.1.8 Molecular-size distribution

The presence of aggregates (which can be reactogenic) and other components in antivenoms can be assessed by size-exclusion liquid chromatography (gel filtration) in HPLC or FPLC systems. Densitometric analyses of chromatographic profiles allow the quantification of protein aggregates and of the relative abundances of: intact immunoglobulins, divalent immunoglobulin fragments (F(ab')2), monovalent immunoglobulin fragments (Fab), dimers, as well as low molecular mass enzymatic digestion products. In intact immunoglobulin-based antivenoms this method allows

quantitation of albumin as its molecular mass (~66 kDa) can be resolved from the ~160 kDa peak of intact immunoglobulins.

15.1.9 Pyrogen test

Antivenoms should comply with the rabbit pyrogen test where required by the local regulations. This test is based on intravenous injection of antivenoms in the ear vein of rabbits (usually 1.0 to 3.0 mL per kg body weight), followed by the measurement of rectal temperature at various time intervals after injection. The detailed procedures are described in various Pharmacopeias. Bacterial lipopolysaccharides can also be detected by the *Limulus* amebocyte lysate (LAL) test. The test should be validated for each type of antivenom, since there have been reports of false positive and false negative reactions when testing antivenoms and other plasma-derived products. The sensitivity of this LAL test should be correlated with the rabbit pyrogen test, and the endotoxin limits established. When regulation allows, a validated LAL test is used in place of the rabbit pyrogen test.

15.1.10 Abnormal toxicity test

The abnormal toxicity test is increasingly abandoned in most regulations as it provides limited information for routine quality assessment of a product. Correct implementation of Good Manufacturing Practices should provide evidence that the product would comply with the test for abnormal toxicity.

15.1.11 Sterility test

Antivenoms should be free of bacteria and fungi, i.e. should be sterile. The sterility test is performed following methodologies specified in various Pharmacopoeias such as the European Pharmacopoeia. Since antivenoms may contain preservatives in their formulation, it is necessary to neutralize the preservatives before the samples are added to culture media. This is usually performed by filtering a volume of antivenom through a 0.45 um pore membrane, and then filtering through the same membrane a solution that neutralizes the bacteriostatic and fungistatic effects of the preservatives used in antivenom. The membrane is then aseptically removed and cut into two halves. One half is added to trypticase soy broth and the other is added to thioglycolate medium. Control culture flasks are included for each medium. Flasks are incubated at 20-25 °C (trypticase soy broth) or at 30-35 °C (thioglycolate) for 14 days. Culture flasks are examined daily for bacterial or fungal growth. The number of vials tested per batch should be in compliance with local regulations.

15.1.12 Concentration of sodium chloride and other excipients

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

15.1.13 Determination of pH

The pH of antivenom should be determined, using a potentiometer.

15.1.14 Concentration of preservatives

When used in the formulation of antivenoms, the concentration of preservatives (phenols, cresols) should be quantified. The acceptable range of preservative concentration in antivenoms should be established and validated in each quality control laboratory. Phenol concentration should not exceed 2.5 g/L and metacresol 3.5 g/L. Phenol concentration can be determined on the basis of the reactivity of phenol with 4-aminoantipyrine, under alkaline conditions (pH 9.0-9.2) in the presence of potassium