- 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 pre-established 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 high-titer 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 half-life. Thus, for viperid envenomings, IgG or $F(ab')_2$ antivenoms have a more suitable pharmacokinetic profile, whereas Fab fragments may be useful for the neutralization of venoms rich in low molecular mass neurotoxins 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 lipid-enveloped 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 reported[1]	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 well-established 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 lipid-enveloped 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 animal-derived antivenoms, in particular taking into account the characteristics of the viruses that may be present in the animal species used to generate antivenoms.

Table 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	Medium-high	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 IgM-enriched	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 equine-derived immunoglobulins (Grandgeorge et al., 1996).

Validation studies showed that heating a purified equine immunoglobulin at 58°C+/-0.1°C without stabilizers inactivates $\geq$4.8 $\log_{10}$ of PRV, $\geq$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 pore-size 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 (tri-n-butyl phosphate or TnBP) at concentrations between 0.3 and 1%, and detergents such as Triton X-100 or Tween 80, also at concentrations generally 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 down-stream 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 disease-associated 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.