Table 7
Viruses identified in horses (*62*, *87*)

Virus	Family	Size (nm)	Genome	Presence in blood reported[a]	Classified as pathogenic to humans (*62*)
Lipid-enveloped viruses					
Borna virus[b]	Bornaviridae	70–130	ss-RNA	Yes	
Equine arteritis virus	Arteriviridae	50–60	ss-RNA		
Equine encephalitis virus, Eastern and Western	Togaviridae	40–70	ss-RNA		Yes
Equine coronavirus	Coronaviridae	75–160	ss-RNA		
Equine foamy virus	Retroviridae	80–100	ss-RNA	Yes	
Equine herpes virus 1–5	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 morbillivirus (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-lipid enveloped viruses					
Equine encephalosis viruses	Reoviridae	80	Ds-RNA		
Equine rhinitis A and B viruses	Picornaviridae	22–30	ss-RNA		
Equine rotavirus	Reoviridae	60–80	Ds-RNA		

[a] Absence of a report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection.

[b] Recent studies have suggested that Borna virus is non-pathogenic to humans (*88*).

Table 8
Viruses identified in sheep and goats (*62*)

Virus	Family	Size (nm)	Genome	Reported presence in blood[a]	Classified as pathogenic to humans (*62*)
Lipid-enveloped viruses					
Adenovirus	Adenoviridae	80–110	ds-DNA		
Akabane virus	Bunyaviridae	80–120	ss-RNA		
Bluetongue virus	Reoviridae	80	ds-RNA		Yes
Border disease virus	Flaviviridae	40–70	ss-RNA		
Borna virus[b]	Bornaviridae	70–130	ss-RNA	Yes	
Bovine herpes virus types 1, 2, 4	Herpesviridae	120–200	ds-DNA		
Bovine viral diarrhoea virus	Togaviridae	40–60	ss-RNA		
Loiping ill virus	Flaviviridae	40–50	ss-RNA		Yes
Nairobi sheep disease	Bunyaviridae	80–120	ss-RNA		
Ovine/bovine papillomavirus	Papillomaviridae	40–55	ds-DNA		
Ovine herpes virus 2	Herpesviridae	120–200	ds-DNA		
Parainfluenza virus type 3	Paramyxoviridae	150–300	ss-RNA		Yes
Peste des petits ruminants (Morbillivirus)	Paramyxoviridae	150–300	ss-RNA		
Poxviruses (Parapox, Capripox, Cowpox)	Poxviridae	140–260	ds-DNA		Yes
Respiratory syncytial virus	Paramyxoviridae	150–300	ss-RNA		
Retroviruses (Caprine arthritis encephalitis virus, Maedi-Visna virus, Jaagsiekte virus, Bovine leukaemia virus)	Retroviridae	80–100	ss-RNA		
Rift Valley fever complex	Bunyaviridae	80–120	ss-RNA		Yes
Ross river virus	Togaviridae	70	ss-RNA		
Rotavirus	Reoviridae	80	ds-RNA		
Tick-borne encephalitis virus	Flaviviridae	40–50	ss-RNA		Yes
Vesicular stomatitis virus	Rhabdoviridae	50–380	ss-RNA	Yes	Yes
Wesselbron virus	Flaviviridae	40–50	ss-RNA		Yes
Non-lipid enveloped viruses					
Epizootic haemorrhagic disease virus	Reoviridae	80	ds-RNA		
Foot and mouth disease virus	Picornaviridae	27–30	ss-RNA		Yes
Reovirus 1-3	Reoviridae	60–80	ds-RNA		

[a] Absence of report does not mean that the virus may not be found in the blood at certain stages of the cycle of infection.

[b] Recent studies have suggested that Borna virus is non-pathogenic to humans (*88*).

Once the step is accurately modelled, 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 sulfate-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 before (e.g. prior to pH adjustment and addition of pepsin) and immediately after (e.g. following 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 as logarithm (log) of the reduction in infectivity that is observed. Total infectivity or viral load is calculated as the infectious titre (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 provides an important indication of the virucidal potential of the step and enables comparison of the data obtained to those from published studies.

Typically, a viral reduction of 4 logs or more 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 adding to 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 an upper pH limit applied to a pepsin digestion or to a caprylic acid precipitation step).

Virus validation studies are subject to a number of limitations (*60*), which should be considered when interpreting the results.

14.3.2 Selection of viruses for the validation of antivenom 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 7 and 8). When possible, viruses known to potentially contaminate animal plasma (called "relevant viruses") should be used.

Table 9 gives examples of a few viruses that have been used for the validation of animal-derived immunoglobulins. Vesicular stomatitis virus (VSV) and West Nile virus (WNV) are relevant lipid-enveloped horse plasma-borne viruses. Bovine viral diarrhoea 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. Encephalomyocarditis virus (EMCV), a picornavirus, can serve as a model for non-lipid-enveloped viruses. Porcine parvovirus can also be selected as a model for small resistant non-lipid-enveloped viruses or as a relevant virus when pepsin of porcine origin is used in the manufacture of $F(ab')_2$ fragments.

This list is not exhaustive 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 9
Examples of laboratory model viruses that can be used for validation studies of horse-derived antivenoms

Virus	Family	Lipid-enveloped	Size (nm)	Genome	Resistance	Model for
Animal Parvovirus (e.g. porcine)	Parvoviridae	No	18–26	SS-DNA	High	Relevant virus (when pepsin of porcine origin is used)
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
Poliovirus; Encephalomyocarditis virus; Hepatitis A virus	Picornaviridae	No	25–30	ss-RNA	Medium-high	Equine rotavirus
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
Sindbis virus	Togaviridae	Yes	60–70	ss-RNA	Low	Eastern, Western and Venezuelan equine encephalitis virus
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

14.4 Viral validation studies of antivenom immunoglobulins

There is no documented case of transmission of zoonotic infections, including viral diseases, by antivenom immunoglobulins, or any other animal-derived immunoglobulins. Absence of reports of viral transmission may result from a lack of long-term surveillance of the patients receiving antivenoms. Alternatively, this may reveal that current processes for the manufacturing of antivenoms include processing steps that contribute to the viral safety.

Among the various processing steps used in the production 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 a production step comprising caprylic acid (*89–91*) or low pH treatment (*60*, *92–94*).

Although information is still limited, there is now growing evidence that similar steps used in the production of animal derived immunoglobulins 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 (*87, 64*) and of human immunoglobulins (*89–91*) are similar, in particular the pH range, duration of treatment, temperature, and the caprylic acid/protein ratio, as summarized in Table 10.

Table 10
Comparison of conditions for caprylic acid treatment used for human immunoglobulin preparations and antivenoms (*87*)

Product	Protein concentration (g/l)	Caprilic acid (g/kg solution)	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	60–90	50	5.5–5.8	18–22	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 lipid-enveloped viruses in human plasma protein fractions (*95, 96*). The non-ionized 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 non-ionized form of caprylate can be maintained over a wide pH range.

The robustness of a caprylic acid treatment applied to human immunoglobulin G (IgG), human immunoglobulin M (IgM) and IgM-enriched preparations has been investigated using various enveloped viruses (human immunodeficiency virus (HIV), BVDV, Sindbis virus and Pseudorabies) (*89*). 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.

Another investigation studied the viral reduction achieved during treatment by caprilate of a human IgG product (*90*). At pH 5.1, at 23 °C, and in the presence of 9 mM caprylate, $\geq$ 4.7 and $\geq$ 4.2 log of HIV and PRV, respectively, were inactivated during the 1-hour treatment, but only 1.5 log for BVDV was inactivated. At 12 mM caprylate, $\geq$ 4.4 log of BVDV were inactivated within this time period. At pH 5.1, 24 °C, and 19 mM caprylate, and pH 5.1, 24 °C, and 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 antivenom immunoglobulins

Virus inactivation studies have been carried out on a $F(ab')_2$ fraction obtained from pepsin digested plasma subjected to ammonium sulfate 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 observed. No significant reduction (0.7 $\log_{10}$) of the non-enveloped EMCV (*97*) was observed.

In another process used to prepare equine immunoglobulins, in which serum is thawed at 4 °C, subjected to 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 of EMCV and Minute virus of mice (MVM) non-lipid enveloped viruses (*98*). 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, robustness studies to define the impact on process variations should also be performed.

14.4.2 Acid pH treatment

The conditions used for low pH treatment of equine derived antivenom immunoglobulins and of human immunoglobulins are summarized in Table 11.

Table 11
Typical conditions for acid pH treatment of human IgG preparations and equine antivenoms (*87*)

Product	Protein concentration (g/l)	pH	Temperature (°C)	Duration (hrs)
Human IgG	40–60	4	30–37	20–30
Antivenoms	60–90	3.1–3.3	30–37	0.6–24

14.4.2.1 Validation studies with human immunoglobulins

Many studies have demonstrated that the low pH 4 treatment used in the manufacture of human intravenous IgG has the capacity to inactivate lipid-enveloped viruses (*92–94*). The rate and extent of inactivation may differ depending upon the virus. Inactivation is temperature-dependent, and is influenced by the formulation of the IgG solution. Pepsin is sometimes added in traces (to reduce anticomplementary activity and content of aggregates) but, at this low concentration, contributes little to virus kill (*60*). Most non-lipid enveloped viruses are resistant to acid pH treatment.

14.4.2.2 Virus inactivation studies performed with antivenom immunoglobulins

As described in section 13, peptic cleavage of horse plasma IgG at pH 3.0–3.3 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 (*99*). WNV was very sensitive whether pepsin was added or not, whereas the rate and extent of inactivation of Sindbis was higher in the presence of pepsin. This suggests that pH 3.2 alone inactivates WNV, while other phenomena involving the action of pepsin contribute 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 obtained by another group (*97*). In this process, plasma is diluted with two volumes of saline, pH is 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. Inactivation of PRV > 5.1 $\log_{10}$ occurred in 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-lipid enveloped viruses may take place during peptic digestion of diluted horse plasma. This does not mean, however, that other non-lipid enveloped viruses would be inactivated to the same extent under such conditions.

14.4.2.3 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. Robustness studies to define the impact on process variations are also recommended.

14.4.3 Filtration steps

Other steps used in antivenom production may contribute to viral safety through non-specific viral removal. The virus removal capacity of two depth-filtration steps performed in the presence of filter aids and used in the production of equine derived immunoglobulins by ammonium sulfate precipitation of pepsin-digested IgG has been evaluated (*100*). Clearance factors of 5.7 and 4.0 $\log_{10}$ have been found for two lipid-enveloped viruses (infectious bovine rhinotraceheitis virus and canine distemper virus, respectively) and of 5.3 and 4.2 $\log_{10}$ for two non-lipid enveloped viruses (canine adenovirus virus and poliovirus type I, respectively). However, it should be kept in mind that viral reductions obtained by non-dedicated removal steps are usually regarded as less robust than those resulting from dedicated viral inactivation or removal steps (*60*).

14.4.4 Validation of dedicated viral reduction treatments

14.4.4.1 Pasteurization

Pasteurization 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 immunoglobulin G (*60*). It is being used in the production process of a few equine-derived immunoglobulins (*10*).

Validation studies showed that heating a purified equine immunoglobulin at 58 °C ± 0.1 °C without stabilizers inactivates ≥ 4.8 $\log_{10}$ of PRV and ≥ 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 MVM, a non-enveloped virus, was still detected after 9 h and 30 min of treatment; only 1.59 $\log_{10}$ were inactivated (*98*).

14.4.4.2 Nanofiltration

Nanofiltration is a technique of filtration specifically designed to remove viruses, based on size, while permitting flow-through of the desired protein (*101*). Effective viral removal requires, in principle, that the pore size of the filter be smaller than the effective diameter of the virus particles.

14.4.5 Other viral inactivation treatments currently not used in antivenom manufacture

Other methods of viral inactivation have been developed to ensure the viral safety of biological products. These 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 between 0.3% and 1%. Such solvent–detergent (S/D) procedures have proven to be very efficient and robust in the inactivation of lipid-enveloped viruses in human plasma products (*60*). However, use of this method for antivenoms has not been reported.

Implementation of dedicated viral inactivation treatments, such as S/D or other methods, should be encouraged for processes which, based on risk assessment, would offer an 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.

14.4.6 Possible contribution of phenol and cresols

The anti-bacterial agents, phenol or cresols, 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 0.25–0.35%. Compounds like phenol are known to be very lipid-soluble and lipophilic.

Performing validations of the potential virucidal effect of antimicrobial agents as added to the starting hyperimmune plasma and to the final antivenom preparations is encouraged. More information is needed on the potential impact of these antimicrobial agents on the viral safety of antivenoms.

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

As there is increasing, although preliminary, evidence that at least some of the existing steps in the production of antivenoms contribute to viral reduction, it is already recommended that specific care should be taken to ensure their appropriate industrial implementation so as not to compromise any possible benefits they provide on viral safety.

Measures should therefore be taken to ensure that such steps are correctly carried out in a manufacturing environment and that cross-contamination and downstream-contamination are avoided. Such important aspects of product safety have been highlighted recently in WHO Guidelines (*60*) and should also be taken into consideration for large-scale manufacture of antivenoms. Specific attention should be given to:

- *Process design and layout*, in particular the production floor area needed to carry out such treatment safely, the justification for creating a safety zone to avoid 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 potential contribution to 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 the homogeneity of mixing cannot be ensured. A poor equipment design could compromise the viral safety potentially 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 performed in two distinct enclosed tanks. Care should be taken to ensure complete process segregation before and after the completion of these treatments to avoid risks of downstream contamination.

- *Process control*: is a critical part of the manufacturing process since completion of viral inactivation and removal cannot be guaranteed by testing the final product. Samples should be taken to confirm that the process conditions of claimed inactivation steps meet the specified limits (e.g. for pH, concentration of stabilizers and 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 the responsibility of the quality assurance department 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. These 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 steps, quality assurance 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

Transmissible Spongiform Encephalopathy (TSE) has not been identified in any 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 and goats, ruminant species used, although less frequently than the 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 scrapie has been experimentally transmitted to monkeys. Because the infectious agents of scrapie and BSE behave similarly in sheep and goats, the use of the blood of small ruminants should either be avoided in preparing biologicals or be selected very carefully from sources known to be free of TSEs. The recent findings of disease-associated proteins in muscle tissue 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 TSE tissue infectivity (*54*).

According to these recommendations, the use of 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 animals assessed to have negligible risk of scrapie. The feed of animals used for production of hyperimmune plasma should be free of ruminant-derived material. Documented surveillance records should be available.

The infectious TSE agents are associated with misfolded, abnormal, prion protein (PrP^{TSE}). It is not yet 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 PrP^{TSE} and infectivity. However, there is still uncertainty about the validity of such experimental studies since PrP^{TSE} has not been detected in blood and plasma and the properties of infectivity in blood are not well understood.

14.7 Main recommendations

- **The viral safety of antivenoms results from a combination of measures:**
  - **to ensure satisfactory health status of the animals;**
  - **to reduce the risk of contamination in the starting material;**
  - **to ensure the contribution of the manufacturing process towards inactivation and/or removal of viruses; and**
  - **to ensure compliance with GMP all along the chain of production.**
- **Manufacturing processes should include at least one, and, preferably, two steps contributing to robust viral reduction. A virus inactivation step that can be easily monitored is usually preferred to other means of viral reduction, such as non-specific removal.**
- **Manufacturers are encouraged to evaluate and validate the capacity of their current manufacturing processes (in particular low pH pepsin digestion, caprylic acid treatment, ammonium sulfate or 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 animals used for the production of the antivenom immunoglobulins.**
- **The removal of antimicrobial agents from the final formulation of antivenoms should be carefully weighed against the potential benefits these agents may have on the viral safety.**
- **Should the viral reduction processes used be found to be 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 analysed in vitro as well as in preclinical studies before performing clinical evaluations in humans.**
- **Great attention should be paid 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-contamination and downstream re-contamination that would jeopardize the viral safety of the product.**
- **When sheeps are to be used for the production of plasma, the animals should be obtained from sources assessed to have negligible risk 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 obtained should meet the specifications approved for each antivenom product or its 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 and shown not to have any impact. The quality control of the final product in antivenoms includes the tests described below.

15.1 Tests

15.1.1 Appearance

The appearance of the product (e.g. colour of the liquid, appearance of the powder) should comply with the description in the marketing dossier.

15.1.2 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 prevent the formation of foam.

15.1.3 Extractable volume

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

15.1.4 Venom-neutralizing potency test

This test determines the effectiveness of the antivenom to neutralize the overall toxic activity of the snake venom(s) against which the antivenom is designed to act. The first part of the test, to determine the lethal activity of the venom, is called the median lethal dose (LD_{50}) assay and usually uses mice of a defined weight range (e.g. 18–20 g). For new venoms whose LD_{50} is unknown, it is recommended that a range dose-finding study, using one mouse per venom dose, is performed to avoid using excessive numbers of animals. Some producers use other test animals, such as guinea-pigs. While weights will clearly vary between animal species, the following principles, specified for mice, will still apply to these alternative test animals. It should be borne in mind that there are variations in the susceptibility of various strains of mice to the lethal effect of venoms.

LD_{50} range-finding test: Various venom doses are prepared using saline solution as diluent, and aliquots of a precise volume (0.2–0.5 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 a precise volume (0.2–0.5 ml) of solutions of varying doses of venom dissolved in sterile saline solution. A minimum of 5 mice is the smallest number recommended for obtaining a statistically significant result. 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 hours (for assays involving intravenous injections) or at 48 hours (when intraperitoneal injections are used), and LD_{50} is estimated by Probit analysis (*102*), Spearman-Karber (*8*) or alternative procedures (such as non-parametric 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} (3–6 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 down 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 3–6 LD_{50}), with various volumes of the antivenom adjusted to a constant final volume with saline solution (*53*, *103*, *104*). The mixtures are incubated for 30 minutes at 37 °C, and then aliquots of a precise volume (0.2–0.5 ml) of each mixture are injected into groups of generally 5 or 6[1] 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 test) or at 48 hours (intraperitoneal test) and the results analysed using Probit analysis (*102*), Spearman-Karber (*8*) or alternative procedures (such as non-parametric 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:

- mg of venom neutralized by ml of antivenom;
- µl antivenom required to neutralize the "challenge dose" of venom used;
- µl of antivenom required to neutralize one mg of venom; and
- number of LD_{50} 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 (i.e. ED_{50}) varies between laboratories and countries, manufacturers should disclose the conditions under which the potency of their antivenoms is estimated to the corresponding regulatory agencies in the course of their licensing and control procedures.

[1] 10 mice may be needed for some venoms.

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 of 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 to ensure that they have not deteriorated (see section 8 on quality control of venoms).

Until in vitro or alternative 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.

15.1.5 Osmolality

Osmolality can be measured to determine the tonicity of the antivenom solution. It is recommended that it be at least 240 mosmol/kg. Determination of osmolality is also an indirect means to determine the quantity of salts or excipients added for formulating the batch.

15.1.6 Identification

When several types of antivenoms are produced by a single laboratory, the identity of each batch of antivenom should be checked. Identity tests may include biological assays as well as physicochemical and immunological tests. Double immunodiffusion assays, confronting the antivenom with the venoms against which the antivenom is designed to act, 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 identify the mammalian species in which the antivenoms are produced. The potency assay against venoms is another way to identify antivenoms.

15.1.7 Protein concentration

The total protein concentration of antivenoms is performed using the Kjeldahl method for nitrogen determination. Alternatively, several colorimetric procedures can be used, as well as measuring absorbance at 280 nm. The presence of preservatives should be taken into account since they may interfere with some protein determination methods (*105*).

The total concentration of proteins in antivenoms should preferably not exceed 10 g/dl, unless a higher protein content is justified and authorized by the competent authority.

15.1.8 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 non-reducing conditions) are suitable for this purpose, since these techniques allow the detection and monitoring of IgG, $F(ab')_2$, Fab, non-immunoglobulin 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% of 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 provides qualitative (or, at best, semiquantitative) information on the amounts of intact immunoglobulins, digestion products and, importantly, on the presence of high-molecular-mass 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 provide information on the amount of immunoglobulins and their fragments by direct visualization of intact and/or digested immunoglobulin heavy chains.

15.1.9 Molecular-size distribution

The presence of aggregates and other components in antivenoms can be assessed by size-exclusion liquid chromatography (gel filtration) in HPLC 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) and 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.

15.1.10 Test for pyrogens

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 ml per kg body mass), followed by the measurement of rectal temperature at various time intervals after injection. The detailed procedures are described in various pharmacopoeias.

Bacterial lipopolysaccharides can also be detected by the *Limulus* amoebocyte 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.

15.1.11 Test for abnormal toxicity test

The abnormal toxicity test may be performed at the stage of product development but is increasingly being abandoned in most regulations as it provides limited information for routine quality assessment of a product. Correct implementation of GMP should provide evidence that the product would comply with the test for abnormal toxicity.

15.1.12 Test for sterility

Antivenoms should be free of bacteria and fungi, i.e. they 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-μm 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.

15.1.13 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.

15.1.14 Determination of pH

The pH of antivenom should be determined using a potentiometer.

15.1.15 Concentration of preservatives

When used in the formulation of antivenoms, the concentration of preservatives (phenol or 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 cresols 3.5 g/l.

Phenol concentration can be determined spectrophotometrically on the basis of the reactivity of phenol with 4-aminoantipyrine, under alkaline conditions (pH 9.0-9.2) in the presence of potassium ferrocyanide as oxidant. Other methods are also available. Cresols can be determined by HPLC methods.

15.1.16 Chemical agents used in plasma fractionation

The chemical reagents used in the precipitation and purification of antivenoms, such as ammonium sulfate, caprylic acid and others, should be removed from the final product during diafiltration or dialysis. Limits should be established and their residual amount quantified in the final product. Likewise, the elimination of pepsin or papain from the final preparations should be guaranteed, especially for preparations that are maintained in liquid form, to avoid proteolytic activity that may damage the antivenoms.

The determination of the residual amount of agents used in plasma fractionation could be excluded from routine release testing if the process of manufacturing has been validated to eliminate these reagents. The detection of residual reagents can also be performed on the final bulk rather than in the final product.

15.1.17 Residual moisture (freeze-dried preparations)

Residual moisture content can be determined by several methodologies, such as:

- a gravimetric method assessing the loss of weight on heating;
- the Karl-Fischer titration, based on the principle that iodine, together with pyridine, sulfur dioxide and methanol from the reagent react quantitatively with water; and
- thermogravimetric methods.

The methodology most commonly recommended is the Karl-Fischer titration. Every manufacturing and quality control laboratory must establish the accepted maximum residual moisture for their antivenom ensuring the stability of the product over its claimed shelf-life. A residual moisture content of less than 3% is usually recommended for most freeze-dried therapeutic biological products.

15.2 Antivenom reference preparations

The use of in-house reference preparations of antivenoms, instead of international standards, is recommended, since the potency and specificity can only be compared with antivenoms of similar specificity and neutralizing profile. An in-house reference preparation should be obtained from a suitable batch of the product that has been fully evaluated by the quality control laboratory..

15.3 Main recommendations

- **Quality control of antivenom preparations, both for product intermediates and final product, as part of the batch release, should be performed by the manufacturers. National regulatory agencies will review the tests performed by the manufacturer and select which tests to develop, when required, on a case-by-case basis.**
- **Quality control tests to be performed by manufacturers as part of the batch release include: neutralization potency test against the most relevant venoms, identification, protein concentration, purity of the active substance, content of protein aggregates and non-IgG contaminants, pyrogen test, sterility test, concentration of excipients, osmolality, pH, concentration of preservatives, determination of traces of agents used in plasma fractionation, visual inspection, and, for freeze-dried preparations, residual moisture and solubility.**
- **Antivenom reference preparations reflecting specific characteristics of antivenoms produced should be prepared by each manufacturer to be used as standards in their laboratory settings, in particular to measure neutralization capacity of their specific antivenom products against targeted venoms. When possible, a national reference antivenom should be established.**

16 STABILITY, STORAGE AND DISTRIBUTION OF ANTIVENOMS

16.1 Stability

Stability studies should be performed to determine the stability of antivenoms. These studies should be done when a new product, a process change, or a new formulation is developed. They are essential to define the shelf-life of the product and are intended to prove that the antivenom remains stable and efficacious until the expiry date.

It has long been considered, somewhat empirically, that liquid preparations have a shelf-life of up to 3 years at 2-8 °C, and freeze-dried preparations up to 5 years, when kept in the dark at room temperature. Nevertheless, the actual stability of each antivenom formulation should be appropriately determined by each manufacturer. It is highly recommended that manufacturers perform stability studies to evaluate the possibility that their preparations could be stored for a long period under non-refrigeration (for instance at 30 °C).

Real-time stability tests should be performed under the expected storage conditions of the antivenom. In addition, these tests could be performed under worst-case storage conditions. Quality control parameters are determined at regular pre-established time intervals. Essential parameters include venom neutralization potency, turbidity and content of aggregates, among others, since these are especially prone to alter upon storage.

Accelerated stability studies may be performed to provide early useful information on the product stability profile, but are not a substitute for real-time data. The antivenom is exposed to

harsher conditions than usual, such as a higher temperature, and the stability is assessed over a shorter timespan.

16.2 Storage

Antivenoms should be stored at a temperature within the range that assures stability, as found by stability tests. This is particularly critical for liquid formulations, which usually require storage at between 2 and 8 °C. Therefore, deviations from this temperature range, due to interruptions in the cold chain during transportation or storage, are likely to result in product deterioration. The design of adequate cold chain programmes, as part of the public health systems in every country, is critical, and national protocols should be developed. The distribution policies for national vaccination programmes can be adopted for the transportation and storage of antivenoms. The stability of liquid preparations at temperatures higher than 2-8 °C should be evaluated and, if needed, new formulations allowing such storage conditions should be developed.

16.3 Distribution

Adequate distribution of antivenoms is a matter of great concern in many regions of the world. Since most of the antivenoms available are liquid preparations, the maintenance of an adequate cold chain must be guaranteed, despite the difficulties to be encountered in rural areas of some developing countries. National and regional health authorities should develop distribution strategies to ensure that antivenoms are allocated to the areas where they are needed or use the distribution channels in place for other national primary health care programmes. Both the specificity of the antivenom and the number of vials or ampoules to be distributed should be taken into consideration. This is particularly relevant in countries that use monospecific antivenoms, since distribution of these products should be guided by the known distribution of the species. To ensure an appropriate supply for clinical use, inventories should be in excess of the estimated number of cases, to allow for unpredictable surges in local demand, accepting that some antivenoms will not have been used by the time of their expiry date.

16.4 Main recommendations

- **The quality control of each antivenom batch prepared by a manufacturer should include the potency test for neutralization of lethality (ED_{50}).**
- **In general, liquid preparations require a cold chain, whereas freeze-dried preparations do not. However, storage conditions are product- or formulation-specific and may vary. Manufacturers should determine the stability of each antivenom pharmaceutical preparation by conducting real-time stability studies.**
- **Manufacturers should study the stability of antivenoms at the ambient temperatures in the areas where the product will be used.**
- **The distribution of antivenoms by health authorities should rely on a proper assessment of the epidemiology of snakebite envenomings, and on the knowledge of the geographical distribution of the most relevant venomous species. This is particularly important for monospecific antivenoms.**
- **National regulatory authorities should ask manufacturers to provide information obtained from the preclinical assessment of all antivenom used in their territories against the venoms found in the region or country where the product is intended to be used.**

17 PRECLINICAL ASSESSMENT OF ANTIVENOMS

17.1 Introduction

A fundamental and ethical requirement of all new therapeutic agents intended for human use is that their safety and efficacy should be established, initially by preclinical in vitro and in vivo laboratory tests and, if the results of these prove satisfactory, by clinical trials in human patients. Information supporting the physicochemical characterization of the new antivenom, such as protein content and level of purity of the preparation should be available before clinical studies are initiated. The assays to be performed are described under section 15, on quality control of antivenoms.

Preclinical testing of antivenoms should be done when:

- a new antivenom is being developed;
- an existing antivenom is to be introduced for use in a new geographical region or country.

In both cases, preclinical studies in animal models should be a regulatory requirement enforced by the medicines regulatory authorities as part of the licensing procedures for antivenoms.

The preclinical tests of new or existing antivenoms necessitate the use of experimental rodents. Despite reservations over the physiological relevance of these animal models to human envenoming and the severity of these in vivo assays (sections 17.4 and 17.5), the tests for determining venom lethality (LD_{50}) and antivenom neutralizing capacity (ED_{50}) are currently the only validated means of assessing venom toxicity and antivenom neutralizing potency by both manufacturers and regulatory authorities worldwide.

It is important to make a distinction between "essential" and "recommended" preclinical assays. The "essential" preclinical assays consist of the overall evaluation of toxic activity of the specific snake venoms (LD_{50}) and the corresponding antivenom neutralizing efficacy of the overall venom(s) toxicity (ED_{50}). These tests are required:

- for the routine quality control of antivenom potency;
- to test the ability of a new antivenom to neutralize the venoms from snakes from the country or region where it is going to be introduced;
- to show neutralizing efficacy of an existing antivenom against medically relevant species in a new geographical region or country.

In summary, before any antivenom is used therapeutically in humans in a given region or country, it should have been preclinically assessed using the "essential" assays against the relevant snake venoms.

Preclinical testing of antivenoms also includes a number of assays whose selection depends on the main pathophysiological effects induced by the venom to be tested. Additional tests are therefore strongly recommended for new antivenoms and for new applications of existing antivenoms to determine whether they are effective in eliminating the most clinically-relevant pathophysiological effects induced by the specific venom(s) of interest.

As an example, a new antivenom developed against *Echis ocellatus* envenoming should be tested for its preclinical neutralizing potency (LD_{50} and ED_{50} tests):

- before it is released for the first time for human trials; and
- for the routine quality control of the potency of subsequent batches.

It is also recommended that the first batch be preclinically tested for its ability to eliminate venom-induced coagulopathy and haemorrhage – the most medically important effects of envenoming by *E. ocellatus*.

17.2 Essential assay for preclinical testing of antivenoms: prevention of lethality

The methodology for estimating the median lethal dose (LD_{50}) of venoms and the median effective dose (ED_{50}) of antivenoms is described in detail in the section on quality control of antivenoms (section 15). The same methods used in the routine quality control of antivenoms should be used in the preclinical testing of all new antivenoms and all new applications of existing antivenoms.

17.3 Additional recommended assays for preclinical testing of antivenoms

It is necessary to test whether antivenoms are effective in the neutralization of the most relevant pathophysiological effects induced by a particular venom. These "recommended" preclinical tests are, however, not intended for the routine quality control of antivenom batches. The relevant methods to be used are listed below.

17.3.1 Neutralization of venom haemorrhagic activity

Many venoms, especially those of vipers, exert powerful local and systemic haemorrhagic activity which is due primarily to venom zinc metalloproteinases. These enzymes damage the basement membrane that surrounds the endothelial cells of capillary blood vessels resulting in bleeding into the tissues. Bleeding into the brain and other major organs is considered to be the major lethal effect of envenoming by many viperid species (*106*). The minimum haemorrhagic dose of a venom (MHD) is defined as the amount of venom (in μg dry weight) which, when injected intradermally, induces in mice a 10-mm haemorrhagic lesion 24 hours after injection (*107*, *108*).

The MHD test is carried out by preparing aliquots of 50 μl of physiological saline solution containing a range of venom doses. Mice (18–20 g body weight; 5 mice per group) are placed under light general anaesthesia (e.g. halothane/oxygen) and the hair surrounding the injection site is shaved. The venom solutions (50 μl) are injected intradermally in the shaved skin. After 24 hours, mice are killed using an approved humane procedure, the area of the injected skin is removed, and the haemorrhagic lesion in the inner side of the skin is measured using calipers in two directions with background illumination. Care should be taken not to stretch the skin. The mean diameter of the haemorrhagic lesion is calculated for each venom dose and the MHD estimated by plotting mean lesion diameter against venom dose and reading off the dose corresponding to a 10-mm diameter (*107*, *108*).

To estimate the ability of an antivenom to neutralize venom-induced haemorrhage, a "challenge dose" of venom is selected, which corresponds to one or more MHDs. Between one and five MHDs have been used as the challenge dose by different laboratories. The test is carried out as above, using 5 mice per group. Mixtures of a fixed amount of venom and various dilutions of antivenom are prepared so that the challenge dose of venom is contained in 50 μl. Controls must include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 min, and aliquots of 50 μl are injected intradermally in lightly anaesthetized mice. The diameter of haemorrhagic lesions is quantified as described above, and the neutralizing ability of antivenom, expressed as MHD-median effective dose (ED_{50}), is estimated as the volume of antivenom, in microlitres, which reduces the diameter of haemorrhagic lesions by 50% when compared with the diameter of the lesion in animals injected with the control venom/saline mixture (*108*).

17.3.2 Neutralization of venom necrotizing activity

Venom-induced local dermonecrosis is a major problem in human victims of snakebite and it has long been considered important to have an assay system to evaluate the effect of an antivenom on this pathology. However, it should be noted that the value of antivenoms in overcoming the cytolytic effects of venoms has not yet been established; indeed, there is considerable doubt whether antivenom is useful in obviating such effects in human victims of snakebite. This is because venom-induced dermonecrosis occurs quickly after a bite and there is usually a considerable delay between the envenoming of a victim and his or her arrival in hospital for treatment. Consequently, antivenom therapy can have little or no effect in reversing the damage (*109*, *110*). Animal experiments in which the antivenom was administered to the animal at different times after the venom support this opinion (*110*, *111*).

The minimum necrotizing dose (MND) of a venom is defined as the least amount of venom (in μg dry weight) which, when injected intradermally into groups of five lightly anaesthetized mice (18–20 g body weight), results in a necrotic lesion of 5 mm diameter 3 days later. The method used is the same as that for the MHD, except that the skin is examined 3 days after the intradermal injection of the venom (*107*).

To estimate the ability of an antivenom to neutralize venom-induced dermonecrosis, a challenge dose of venom is selected, usually between one and two MNDs. The test is carried out as above, using 5 mice per group. Mixtures of a fixed concentration of venom and various dilutions of antivenom are prepared so that the venom challenge dose is contained in 50 μl. Controls include venom solutions incubated with physiological saline solution alone. Mixtures are incubated at 37 °C for 30 min, and aliquots of 50 μl are injected intradermally in lightly anaesthetized mice (*112*, *113*). The diameter of dermonecrotic lesions is quantified 3 days after injection, as described above, and the neutralizing ability of antivenom, expressed as MND-median effective dose (ED_{50}), is estimated as the volume of antivenom, in microlitres, which reduces the diameter of necrotic lesions by 50% when compared with the diameter of the lesion in mice injected with the control venom/saline mixture.

17.3.3 Neutralization of venom procoagulant effect

Many venoms, especially from some vipers, cause consumption of coagulation factors which results in incoagulable blood. This, combined with the haemorrhagic nature of some of these venoms, can result in a very poor prognosis for a severely envenomed patient. Simple in vitro methods exist to measure this venom-induced pathophysiological effect and the ability of an antivenom to eliminate it.

The minimum coagulant dose (MCD) of a venom is defined as the least amount of venom (in mg dry weight per litre of test solution or μg/ml) that clots either a solution of bovine fibrinogen (2 g/l) in 60 sec at 37 °C (MCD-F) and/or a standard citrated solution of human plasma (fibrinogen content 2.8 g/l) under the same conditions (MCD-P).

For measurement of the MCD-F, 50 μl of physiological saline with final venom concentrations ranging from 240 to 0.5 mg/l is added to 0.2 ml of bovine fibrinogen solution at 37 °C in new glass clotting tubes. The solutions are mixed thoroughly and the clotting time recorded. The MCD-P is estimated by adding the same venom concentrations to 0.2 ml of the standard human plasma solution under identical conditions and recording the clotting time. In each case, the MCD is calculated by plotting clotting time against venom concentration and reading off the level at the 60-second clotting time (*107*).

To estimate the ability of an antivenom to neutralize venom procoagulant activity, a challenge dose of venom is selected, which corresponds to one MCD-P or one MCD-F. Mixtures of a fixed