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Mini-review

Assessment of the viral safety of antivenoms fractionated from equine plasma

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Abstract

Antivenoms are preparations of intact or fragmented (F(ab')₂ or Fab) immunoglobulin G (IgG) used in human medicine to treat the severe envenomings resulting from the bites and stings of various animals, such as snakes, spiders, scorpions, or marine animals, or from the contact with poisonous plants. They are obtained by fractionating plasma collected from immunized horses or, less frequently, sheep. Manufacturing processes usually include pepsin digestion at acid pH, papain digestion, ammonium sulphate precipitation, caprylic acid precipitation, heat coagulation and/or chromatography. Most production processes do not have deliberately introduced viral inactivation or removal treatments, but antivenoms have never been found to transmit viruses to humans. Nevertheless, the recent examples of zoonotic diseases highlight the need to perform a careful assessment of the viral safety of antivenoms.

This paper reviews the characteristics of equine viruses of antivenoms and discusses the potential of some manufacturing steps to avoid risks of viral contamination. Analysis of production parameters indicate that acid pH treatments and caprylic acid precipitations, which have been validated for the manufacture of some human IgG products, appear to provide the best potential for viral inactivation of antivenoms. As many manufacturers of antivenoms located in developing countries lack the resources to conduct formal viral validation studies, it is hoped that this review will help in the scientific understanding of the viral safety factors of antivenoms, in the controlled implementation of the manufacturing steps with expected impact on viral safety, and in the overall reinforcement of good manufacturing practices of these essential therapeutic products.

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1. Introduction

Antivenoms are important biopharmaceutical products made from the plasma of immunized horses or sheep. They are used in human medicine to treat the potentially severe pathophysiological complications resulting from the bites and stings from various animals, such as snakes, scorpions, spiders, cnidarians, lepidopterans or fishes, as well as from intoxications with plants [1,2]. Envenomings are a serious health problem worldwide and are most particularly dreadful in rural areas of the developing world [3], where shortage of antivenom products [4] and lack of sufficient medical facilities explain numerous fatalities [5,6].

Snake bites represent the major cause of envenoming. Case fatality associated with some snake bites reach 50% or more but can be reduced to less than 5% through antivenom therapy, the only available current

treatment [5,7–9]. Global mortality from snake bites may range from 50,000 to 100,000 per year, but these figures are likely underestimated [5,10]. Viperid snake bite envenoming induces local effects, such as swelling, pain, necrosis, hemorrhage and blistering, often accompanied by secondary infection [9]. Systemic viperid envenoming is characterized by a complex pathophysiological profile including coagulopathy, hemorrhage, hypovolaemia, shock and acute renal failure [9,11]. Progressive paralysis may be caused by elapid snake venom neurotoxins, and by some viperid venoms displaying neurotoxic effects. Some elapid venoms also induce local necrosis and rhabdomyolysis [9]. Snake bite survivors may have major chronic physical and neurological disability.

Scorpion stings are the second major cause of human fatalities from envenoming (probably amounting to several hundreds per year). Scorpion venoms contain

toxins which target sodium, potassium, calcium and chloride channels, causing direct effects and release of neurotransmitters such as acetylcholine and catecholamines, inducing intense local pain and potentially fatal neurotoxic and hemodynamic disturbances [12]. The role of antivenom in the treatment of scorpion stings and other arachnids remains controversial but several reports support their manufacture and use, particularly in severe cases [13,14].

Fatalities have also occurred from envenoming by jellyfish, and venomous fishes. Long-term anecdotal experience supports the beneficial effect of stonefish antivenoms [2], but those may need to be given very early to fight a rapid onset of cardiotoxicity. Stings by cnidarians, lepidoptera, centipedes and coneshells and bites by spiders, ticks and one genus of octopus, probably account for about 100 deaths per year [5]. Finally, envenomings by massive attacks of Africanised bees cause some 30 deaths each year in the Americas [5].

Antivenoms are most often made by fractionation of plasma of horses (less frequently sheep) that have been immunized with crude venoms [1]. Pooled hyperimmune plasma is processed to purify the horse immunoglobulin G (IgG) fraction that may be subjected to enzymatic treatment to obtain F(ab')₂ and Fab antibody fragments, and caprylate or ammonium sulphate precipitation to improve their purity [1]. To some extent, some manufacturing steps have features similar to those used to prepare human plasma-derived IgG. Although there is no known transmission of any infectious agent through antivenoms (albeit under circumstances where rigorous clinical patient follow-up is difficult), theoretical concerns about the possibility of transmission of horse/sheep infectious agents to humans do exist. Recent natural transmissions of zoonotic diseases highlight the possible exchanges between humans and the natural reservoirs of biologic agents found in animals [15], and the inherent risk of emerging diseases [16]. Examples of such infections originating from animal (or avian) pathogens include human immunodeficiency virus, Ebola, Hantaan, Lassa, Nipah viruses and other paramyxoviruses, equine morbilli virus, West Nile virus, and probably severe acute respiratory syndrome (SARS) coronavirus [17,18]. The parenteral transmission of animal viruses to humans is also possible. Infectious SV40 virus of rhesus monkeys was a contaminant of early polio vaccines which were administered to a large number of people. Whether this was the way in which this virus was introduced into the human population is unclear and a controversial issue.

The risk of contamination of antivenoms by equine virus is, therefore, of theoretical concern and has been debated at a recent World Health Organization (WHO) workshop [5]. Recently, the Committee for Proprietary Medicinal Product (CPMP) of the European Medicine Evaluation Agency has published a Note for Guidance

on the manufacture and quality control of animal immunoglobulins and immunosera [19]. However, most manufacturers of antivenoms are located in the developing world and some of them may not be directly exposed to the state-of-the-art process validation concepts, nor have the financial and logistics capability to perform extensive viral validation studies to assess the viral reduction potential of the process they use. Therefore, we found it helpful to examine the viral safety of antivenoms by first reviewing the characteristics of equine viruses. We then evaluated the theoretical ability of manufacturing methods of antivenoms to inactivate or remove those viruses, through a comparison with the well validated technologies used to manufacture human plasma-derived IgG preparations. Finally, we want to emphasize that applying good manufacturing practices in the production of antivenoms, from the control of the animals to that of all production steps, and ensuring traceability in the whole chain of manufacture, represent the current best investment in the quality and safety of these products.

2. Equine viruses

Horses can harbour enveloped and non-enveloped viruses. The main structural characteristics of those viruses are presented in Table 1; epidemiological and clinical data and testing methods are described briefly below.

2.1. Enveloped viruses

2.1.1. Arteritis virus [EAV]

EAV is a 50–60 nm single-stranded (ss)-RNA virus of the *Arteriviridae* family [20]. EAV causes an equine viral arteritis, an endotheliotropic viral disease [21]. Transmission occurs via respiratory and reproductive routes. There is a variety of clinical signs, and strains vary in virulence [21], but severe infection can lead to abortions in pregnant mares or neonatal foal death. A one-tube real-time TaqMan RT-PCR assay was developed for detecting EAV. The test was validated using seminal plasma and nasal secretions [22]. A DNA vaccine was shown to induce long-term immunization [23].

2.1.2. Borna virus [BDV]

BDV is a 70–130 nm ss-RNA virus belonging to the new *Bornaviridae* family, order Mononegavirales. Borna disease, known as ‘disease of the head’, is a sporadically occurring, progressive viral polioencephalomyelitis that primarily affects horses and sheep. After a few weeks to several months incubation, BDV can cause locomotor and sensory dysfunction followed by paralysis and death. BDV exists world-wide in horses, sheep, cattle,

Table 1
Viruses identified in horses

Virus	Family	Size (nm)	Genome	Reported presence in horse blood	Classified as pathogenic to humans ^a
<i>Enveloped</i>					
Arteritis virus	<i>Arteriviridae</i>	50–60	ss-RNA	NR	–
Borna virus	<i>Bornaviridae</i>	70–130	ss-RNA	Yes	Yes
Eastern & western equine encephalitis virus	<i>Togaviridae</i>	40–70	ss-RNA	NR	Yes
Equine coronavirus	<i>Coronaviridae</i>	75–160	ss-RNA	NR	–
Equine foamy virus	<i>Retroviridae</i>	80–100	ss-RNA	Yes	–
Equine herpes virus 1-5	<i>Herpesviridae</i>	125–150	ds-DNA	NR	Yes
Equine infectious anaemia virus	<i>Lentiviridae</i>	80–100	ss-RNA	Yes	–
Equine influenza virus	<i>Orthomyxoviridae</i>	80–120	ss-RNA	NR	Yes
Equine morbilli virus (Hendra virus)	<i>Paramyxoviridae</i>	150	ss-RNA	NR	Yes
Japanese encephalitis virus	<i>Flaviviridae</i>	40–70	ss-RNA	NR	Yes
Nipah virus	<i>Paramyxoviridae</i>	150	ss-RNA	NR	Yes
Salem virus	<i>Paramyxoviridae</i>	150	ss-RNA	NR	–
St-Louis encephalitis virus	<i>Flaviviridae</i>	40–70	ss-RNA	NR	Yes
Venezuelan equine encephalitis virus	<i>Togaviridae</i>	40–70	ss-RNA	Yes	Yes
Vesicular stomatitis virus	<i>Rhabdoviridae</i>	50–380	ss-RNA	Yes	Yes
West Nile virus	<i>Flaviviridae</i>	40–70	ss-RNA	Yes	Yes
<i>Non-enveloped</i>					
Equine encephalosis	<i>Reoviridae</i>	80	ds-RNA	NR	–
Equine rhinitis A and B viruses	<i>Picornaviridae</i>	22–30	ss-RNA	NR	–
Equine rotavirus	<i>Reoviridae</i>	60–80	ds-RNA	NR	–

NR = not reported based on literature search but cannot be excluded.

^a Based on the recent EMEA note for guidance [19].

cats, dogs and ostriches and can affect a large number of warm-blooded animal species, including humans [24,25]. The infection can be fatal, but the majority of carriers are asymptomatic [26]. Cross-species transmission of this commensal virus has not been proven, but zoonotic aspects of BDV should be considered [26]. BDV-specific antibodies and viral RNA have been found in humans with various psychiatric disorders. Diagnosis can be made serologically, but also by antigen markers in peripheral white blood cells, combined with nucleic acid amplification [26].

2.1.3. Eastern, western, and Venezuelan equine encephalitis alphavirus (EEEV, WEEV, VEEV)

These are closely related 40–70 nm ss-RNA alphaviruses [arbovirus type A] of the *Togaviridae* family that are transmitted by arthropods (usually mosquitoes). Symptoms of EEEV infections, an important multi-systemic zoonotic disease, include anorexia and colic, changes in sensorium, hyperexcitability, and terminal severe depression. Organ coagulative necrosis and CNS lesions are observed [27]. WEEV is not as neuroinvasive as EEEV and the encephalitis caused is not as severe as that caused by EEEV. A test to detect EEEV and WEEV viral RNA has recently been developed [28]. VEEV is present in both humans and horses but no evidence of transmission from horses to humans by normal routes

of contamination has been found. VEEV has been identified in pharyngeal secretions and is stable when aerosolized; it has been shown to be stable in dried blood and exudates. Vaccines have been developed against those three viruses [29]; one possible case of viral transmission in horse has been suspected following vaccination [30].

2.1.4. Equine coronavirus [ECV]

ECV is a 75–160 nm ss-RNA virus of the *Coronaviridae* family. It has been isolated from feces of a diarrhoeic foal, and has close antigenic and/or genetic relationships with mammalian group 2 coronaviruses [31].

2.1.5. Equine foamy virus [EFV]

EFV, also known as spumavirus, is a 80–100 nm ss-RNA virus of the *Retroviridae* family. It belongs to the nonpathogenic, complex unconventional retroviruses and has been isolated in nonhuman primates, cattle, cats, and more recently in the blood of horses [32].

2.1.6. Equine herpes viruses [EHV]

EHVs are large 125–150 nm double-stranded (ds) DNA gamma herpes viruses. EHV1 and EHV4 cause much damage to the horse industry and are ubiquitous in the equine population. They are responsible for

life-long latent infections in their hosts even in those with natural or vaccine-induced immunity [33]. EHV1 strains are associated with respiratory disease, abortion, and paresis/paralysis, whereas EHV4 strains induce respiratory disease [34]. EHV2 and EHV5 have a less clear pathogenicity and distribution within the equine population. EHV2 may have an aetiological role in ocular disease [35]. The prevalence of EHV2 in adult horses was found to be up to 68% in Sweden and 71% in the United Kingdom. The prevalence of EHV5 DNA was 15 and 24% in adult horses in Hungary and the United Kingdom, respectively [36].

2.1.7. *Equine infectious anaemia virus [EIAV]*

EIAV is a 80–100 nm ss-RNA lentivirus of the same *Retroviridae* family as HIV. Most infected horses are asymptomatic, with life-long latent infection of leucocytes until stressed (e.g. by pregnancy, corticosteroids, surgical operation, disease) or until new virus variants arise. Viraemia then increases by 100-fold. Red blood cells become coated by the virus particles and are then lysed by complement, causing jaundice, oedema, hemorrhagic diarrhoea, petechial hemorrhages. EIAV can be transmitted from horse to horse by blood. Recently a reverse-transcriptase polymerase chain reaction assay (RT-PCR) has been described for quantifying EIAV RNA in equine plasma [37].

2.1.8. *Equine influenza virus [EIV]*

EIV is a 80–120 nm ss-RNA virus of the *Orthomyxoviridae*. Equine influenza is one of the most economically important contagious respiratory diseases of horses [38].

2.1.9. *Equine morbilli virus (Hendra) [HeV]*

HeV is a 150 nm ss-RNA virus [39]. The virus is probably transmitted from bats to horses, and causes natural disease in humans and horses. It is the reference virus for a proposed new genus within the *Paramyxoviridae* family, which also includes another newly identified zoonotic bat virus (Nipah), and the Salem virus. HeV has been recognized in Australia as a new zoonotic disease of horses since 1994/1995. This lethal zoonotic viral agent is endemic in certain species of fruit bats (flying-foxes) and over 20% of them in eastern Australia are seropositive [40]. Six species of flying-foxes in Papua New Guinea have tested positive for antibodies to HeV [40]. HeV does not appear to transmit readily between horses under natural and experimental conditions, or from horses to humans [41]. Horses can be infected by oronasal routes and can excrete HeV in urine and saliva [41]. The virus appears to be spread by close contact with body fluids, such as froth from infected lungs [42], via nasal discharge, saliva and/or urine [43]. The most important clinical and pathological manifestation of HeV infection in horses and humans is

severe interstitial pneumonia caused by viral infection of small blood vessels. From 1994 to 1999, epizootic and epidemiological episodes of meningoencephalitis and severe acute respiratory syndromes were reported in Australia, Malaysia and Singapore [15,44]. It may also cause nervous disease. Three incidents of HeV disease in horses have been recorded in Australia—two in 1994, which caused the death of two humans and 15 horses and one in 1999, which involved the death of a single horse [45]. Infected horses can develop a severe and often fatal respiratory disease characterized by dyspnoea, vascular endothelial damage and pulmonary oedema. Nervous signs may also occur. A number of diagnostic methods have been developed [46], based on the examination of blood, lung, kidney, spleen, and, if nervous signs are present, also of the brain. PCR assays have been developed. A rapid and sensitive RT-PCR assay using a fluorogenic (TaqMan) probe was developed to improve the diagnosis of HeV infection [47].

2.1.10. *Japanese encephalitis virus [JEV] and Saint-Louis encephalitis virus [SLEV]*

Both are closely related, arthropod-borne, 40–70 nm ss-RNA flaviviruses of the *Flaviviridae* family that may affect the central nervous system of horses [48,49].

2.1.11. *Nipah virus [NiV]*

Evidence of infection with NiV was found in the brain of one horse in which inflammation of the meningeal blood vessels occur [42]. NiV emerged in Malaysia, spread rapidly through the pig population, and caused the deaths of over 100 people [39].

2.1.12. *Salem virus [SalV]*

SalV has been recently identified in horses [50]. The only known isolate was obtained from a horse that was involved in a disease outbreak of undetermined nature [51].

2.1.13. *Vesicular stomatitis virus [VSV]*

VSV is a 50–380 nm ss-RNA virus of the *Rhabdoviridae* family. It may cause stomatitis in horses, and may represent an emerging equine infectious disease [52]. An immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MC-ELISA) has been developed for the detection of primary infection of VSV in equine and swine sera [53].

2.1.14. *West Nile virus [WNV]*

WNV is a 40–70 nm ss-RNA arbovirus of the *Flaviviridae* family, first identified in the West Nile district of Uganda in 1937. WNV is found over a broad geographical range and in a wide diversity of vertebrate host and vector species [54]. Until 1999, the disease was found in African, European, and Eurasian countries.

More recently, there was an increase in outbreaks of illness due to WNV in animals as well as humans and numerous cases are currently reported in Canada and the USA. Infected humans may be asymptomatic and the transfusion of donated blood from such individuals has resulted in the infection and death of recipients. This has led to the introduction in 2003 of strict regulatory measures in Canada and the USA which include the testing by a PCR assay of all blood donations. WNV primarily circulates between birds and mosquitoes, while mammalian infections are incidental. Mammal biting mosquitoes become infected when they feed on the blood of an infected bird. Once this happens, people, horses and other mammals may get WNV. Infected horses are, however, unlikely to serve as important amplifying hosts for WNV in nature [55]. Many WNV-infected horses probably never show symptoms, but a study reported mortality rates close to 30% [56]. Early symptoms are often indistinguishable from other equine encephalitis including rabies, equine herpesvirus-1, equine protozoal myeloencephalitis, and eastern, western, or Venezuelan equine encephalomyelitis. A vaccine for horses has been developed, but one horse that received two doses died from the disease [56].

2.2. Non-enveloped viruses

2.2.1. Equine rhinitis A and B viruses [ERAV and ERBV]

ERAV and ERBV are picornaviruses reclassified as members of the Aphovirus genus because of resemblance to foot-and-mouth disease virus. They are 22–30 nm ss-RNA viruses. High neutralizing antibody titers develop and appear to correlate with strong reactivity to VP1 in Western blots [57]. A new serotype of the genus Erbovirus, tentatively named ERBV2 has been identified recently and found in 24% of horses in Australia [58].

2.2.2. Equine encephalosis virus [EEV]

EEV is a 80 nm ds-RNA virus of the *Reoviridae* family. Several serotypes have been identified in southern Africa. Serotype-specific virus-neutralizing antibody in serum samples from horses suggests a widespread occurrence of infection but there seems to be only a low level of cross-protection in horses to natural reinfection [59].

2.2.3. Equine rotavirus [ERV]

ERV is a 60–80 nm double-stranded RNA of the *Reoviridae*. Rotaviruses are important pathogens associated with diarrhoeal diseases in almost all species of mammals. A PCR test with a detection limit of approximately 1.6×10^2 TCID₅₀ per gram faeces, with possible increase in the sensibility by one order of

magnitude using nested PCR, was developed, representing a possible diagnostic tool [60].

3. Production methods of antivenoms

Antivenoms are produced from the plasma or serum collected from immunized horses or sheep. Plasma can be obtained by the centrifugation of whole blood or by apheresis. Plasma from several animals is typically pooled into 20–300 l batches and subjected to a fractionation process to isolate the IgG fraction. Following the initial use of crude equine immune serum, still reported to be manufactured by one producer [5], various methods of IgG purification and refinement have been introduced (Table 2). Most manufacturers use protocols derived from the basic method described by Pope [61,62], modified by Harms [63], based on pepsin digestion at low pH, to obtain F(ab')₂ fragments, followed by ammonium sulphate precipitation of antibody fragments [5,64,65]. This basic approach is combined with a number of additional steps, aimed at obtaining a purer [66] preparation, such as heat coagulation [64] and ion-exchange chromatography [66,67]. The pH at which pepsin digestion is performed by various laboratories ranges between 3.1 and 3.8, at temperatures between 30 and 37 °C. Pepsin digestion is usually performed in undiluted serum or plasma (protein concentration: 60–90 g/l). Incubation times range from 40 min to 24 h and with varying pepsin concentrations [64,65,67,68]. Thermocoagulation is used by many laboratories and consists of heating at 55–56 °C for 1 h [64,65,68] although not all methods involving pepsin digestion include heat coagulation [67]. Some fractionation protocols include an additional

Table 2
Steps used for the manufacture of antivenoms

Screening of production animals for adventitious agents
Plasma collection (whole blood or apheresis) in bags or bottles
Plasma thawing at room temperature
Plasma pooling
IgG/fragments purification process
<ul style="list-style-type: none"> • F(ab')₂: <ul style="list-style-type: none"> ○ pepsin digestion (acid pH) and ammonium sulphate precipitation, or ○ pepsin digestion (acid pH) and caprylic acid precipitation • Fab: ammonium sulphate precipitation and papain digestion at pH 7–8 • Whole IgG: <ul style="list-style-type: none"> ○ caprylic acid precipitation ○ ammonium sulphate precipitation
IgG/fragments concentration: (NH ₄) ₂ SO ₄ /Na ₂ SO ₄ precipitation, ultrafiltration
Polishing: ion exchange (removes Fc and further purifies IgGs/fragments)
Ultrafiltration
Sterile filtration
Aseptic filling
Storage in the liquid state or lyophilisation

acidification step to remove some non-IgG globulins ('euglobulins') which are unstable at acid pHs [66,69]. One producer includes a bulk pasteurisation step in its antivenom production protocol [66].

Other manufacturers produce whole IgG antivenoms using either ammonium sulphate precipitation of IgG or caprylic acid precipitation of non-IgG proteins [69–71], followed by dialysis or ultrafiltration. Recently, a simple, one-step methodology based on caprylic acid precipitation has been described [72]. The conditions used for caprylic acid fractionation of equine plasma are as follows: plasma pH is adjusted to 5.8 and caprylic acid is added directly to this plasma to attain a final concentration of 5% (v/v). The mixture is stirred during caprylic acid addition and then for one additional hour, after which the precipitate is separated by filtration. Caprylic acid is then removed by either dialysis or ultrafiltration and the product is formulated before sterile filtration [72]. Some antivenoms are made of Fab antibody fragments, obtained by papain digestion of sheep IgG, at neutral pH, after sodium sulphate precipitation of IgG [73]. Affinity-chromatography or ion-exchange chromatography steps have been described in the production of some Fab antivenoms [74,75]. Treatment with β -propiolactone has been evaluated to reduce complement activation by horse plasma-derived products [76]; however, it does not appear to be used routinely in laboratories producing antivenoms.

In addition to the differences in fractionation protocols, antivenoms made of whole IgG, F(ab')₂ and Fab molecules greatly differ in their pharmacokinetic profiles [1,77]. Most antivenoms come in liquid presentation, but some are lyophilized. The former need to be stored at 4–8 °C, since higher temperatures reduce their shelf-life and induce protein denaturation and aggregate formation [78]. In contrast, lyophilized antivenoms can be stored at room temperature and have a more prolonged shelf-life. Most antivenoms contain preservatives to prevent bacterial contamination [5,79].

4. Application to antivenoms of experience from human plasma products

Considerable experience has been gained in recent years on the viral safety of human IgG preparations and other human plasma-derived products. Historical perspectives on these products help in assessing the viral safety of antivenoms. The safety of human plasma products results from the combination of overlapping strategies that include: (a) selection of donors, (b) viral testing procedures on single donations (e.g. serological tests) and plasma pools (e.g. nucleic acid tests) to exclude donations contaminated by viruses, (c) processing and purification steps to inactivate and remove viruses, and (d) implementation of good manufacturing

practices [80]. Whenever feasible, similar approaches should be used for the manufacture of animal plasma-derived products.

4.1. Monitoring of animals used in antivenom production

Monitoring the health of animals used for antivenom production is important [5]. Colonies of animals should be kept free of contamination through good husbandry or vaccination, where appropriate. Surveillance of the source animals should include screening for pathogens, health monitoring, including routine blood chemistry and haematology tests, and post-mortem examination. Ideally, animals should be kept under strictly contained conditions, but this is not readily applicable to horses. However, for instance, animals could be kept free of a particular arthropod-borne virus if they are maintained in an area free of the particular arthropod vector. If a particular pathogen is identified, it should be established whether it is present in plasma. Some virological screening methods may be performed, if available and epidemiologically relevant to a particular geographic region, to limit risk of virus presence in the animal herd and/or the plasma pool. To some extent the Guidelines from the Federation of European Laboratory Animal Science Association (FELASA), although not referring to horses, may serve as a reference [81–83]. Nevertheless, not all of these measures are available in many developing countries nor readily applicable to horses used for antivenom production, and pathogens can escape this surveillance program. This illustrates the need for manufacturing techniques to ensure a sufficient margin of safety with regards to potential infectious agents present in the manufacturing plasma pool. This also strongly emphasizes the crucial importance of ensuring an appropriate traceability system from horse donors to human recipients of antivenoms so that any potential infectious events or risks may be identified in a proper and timely manner and relevant counter-measures taken promptly.

4.2. Viral inactivation treatments

Confidence in the safety of antivenoms must come from evidence that the manufacturing processes used can reproducibly remove or inactivate high potential levels of viruses. Comparison with human plasma fractionation suggests that some of the steps of antivenoms include treatments which may inactivate viruses. Human plasma fractionation is a complex integrated manufacturing process from which products like albumin, coagulation factors and IgG, are obtained. Purification processes combine cryoprecipitation, ethanol fractionation, chromatography, and

ultrafiltration [84]. In addition, various types of dedicated viral reduction treatments have been introduced, most often on purpose, such as chemical treatments by solvent-detergent, caprylic acid precipitation, low-pH incubation, heat treatments in the liquid or in the dry states, and nanofiltration [80]. Among those, caprylic acid and low pH treatments, both of which are commonly used also for the purification of antivenom IgG, have been shown to contribute to the viral safety of human plasma IgG products as described below.

4.2.1. Caprylic acid treatment

Some years ago, unsaturated fatty acids were shown experimentally to inactivate more than 4 log of lipid-enveloped viruses (VSV, Sindbis virus, HIV) in human plasma protein fractions [85]. However, specific attention has been given to caprylic acid (also called octanoic acid), as it has been used as precipitating agent for human IgG. It has been suggested that the non-ionized form of the caprylic acid disrupts the integrity of 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. Treatment of plasma protein solution with caprylate at acidic pH also readily inactivates lipid-enveloped viruses like herpes simplex virus type I, vesicular stomatitis virus, vaccinia virus, and Sindbis virus [86].

Detailed validation studies of two caprylic acid treatments of human Ig have been published recently. The robustness of a treatment applied to three intravenous immunoglobulin preparations (IgG, IgM-enriched, and IgM preparations) has been investigated using HIV, BVDV, Sindbis virus and pseudorabies as model viruses [87]. The routine treatment conditions for these two IgG and this IgM preparation are indicated in Table 3. Kinetics of inactivation was determined over a period of 1 h of treatment. Complete inactivation (corresponding to more than 4.68–6.25 log) is achieved within the first minutes. Within a certain range, viral inactivation in the IgG product 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 EAV, an equine virus used as a model (Dichtelmüller, personal communication). Another study reported the viral inactivation achieved during caprylic acid precipitation of non-IgG proteins from human IgG product [88]. 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 1 h treatment, but only 1.5 log for BVDV was inactivated. At 12 mM caprylate, ≥ 4.4 log of BVDV was inactivated within this time period. At pH 5.1, 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. The authors also showed that 40 mM caprylate at pH 5.4 and 40 °C in supernatant IV-1, an intermediate in albumin production, inactivated ≥ 4.2 log BVDV almost instantaneously [88].

The virucidal effect of caprylic acid has also been confirmed in human albumin solution, where it is used as a stabilizer for pasteurisation. Elevated temperature and low pH were found to be critical parameters to ensuring significant reduction in virus infectivity. Rate and extent of inactivation were sensitive to variations in the caprylate to protein ratio and to changes in pH. In the conditions retained, 10% w/v protein, 16 mM caprylate, pH 4.5 and 30 °C, more than 4 log inactivation of the lipid-enveloped viruses tested, including BVDV, was achieved [89].

It should be kept in mind that treatment of whole plasma or crude fractions, as is the case for equine antivenoms production, may lead to lower rate and kinetics of viral inactivation, due to the high endogenous lipid content, as found in a study that evaluated the virucidal effect of sodium oleate [85]. Finally, one should keep in mind that caprylic acid treatment does not inactivate non-enveloped viruses.

4.2.2. Acid pH treatment

Several human IgG preparations are subjected to an acid pH incubation that was historically introduced to reduce immediate adverse reactions subsequent to intravenous injections. In the late 1980s, acid pH treatment was also shown to contribute greatly to the

Table 3
Comparison of conditions for caprylic acid treatment used for human IgG preparations (87) and equine antivenoms

Product	Protein concentration (g/l)	Caprylate/kg solution (g)	pH	Temperature (°C)	Duration (h)
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
Equine IgG	60–90	50	5.5–5.8	18–22	1

viral safety of human IgG preparations [90,91]. The treatment of human IgG generally consists of incubation at pH 4, with or without traces of pepsin, at temperatures from 30 to 37 °C, using a protein content close to 50 g/l, and for more than 20 h. The treatment is intended to eliminate aggregates. Pepsin, when present, is maintained at low concentration to avoid cleavage of the IgG molecule. Several model enveloped viruses were shown to be inactivated under those conditions [92–94]. More than 5–8 log inactivation of HIV and other enveloped viruses used as surrogate models, such as Semliki forest virus (SFV), HSV, VSV, and CMV is achieved. By contrast, poliovirus, as other non-enveloped virus, seems more resistant. The presence of trace concentrations of pepsin added to reduce anticomplementary activity during this procedure has been shown to contribute little to virus kill of most processes [95]. Virus inactivation by acid pH and pepsin is influenced by several parameters. HIV, BVDV, SFV, and PRV are completely inactivated within 5 min–1 h at 37 °C but inactivation rate and extent is less at lower temperatures. Increasing the sucrose content from 0 to 15% reduced the rate of inactivation of PRV but not that of SFV. Increasing the NaCl content from 0 to 150 mM reduced the rate of inactivation of SFV but that of PRV was unchanged. An increase in the IgG concentration from 0 to 10% speeds up the inactivation of PRV but decreases that of SFV. Therefore, temperature is a major parameter in the virucidal efficacy of pH-pepsin treatment and the impact of solute composition is virus-dependent [93]. Some fractionation protocols used in antivenom production include a low pH treatment to induce the precipitation of ‘euglobulins’, which are plasma globulins unstable under these conditions. This step was shown to remove between 2.8 and 3.5 log of EIAV, Sindbis virus, poliovirus and porcine parvovirus [66]. Virus reduction may occur by co-precipitation with the discarded ‘euglobulins’ rather than by an inactivation process [66].

4.2.3. Liquid heat treatment

Pasteurisation is the treatment of a liquid protein fraction for 10 h at 60 °C [96] and is being used in the production of at least one type of equine-derived antivenom [66]. Experience with human plasma products show that liquid heat treatment may inactivate both enveloped and non-enveloped viruses. Pasteurisation of albumin solutions is carried out in the final container in the presence of low concentrations of sodium caprylate alone or with *N*-acetyl tryptophan. Inactivation of Sindbis and EMC model viruses added to 5% albumin solution was achieved within 10 min treatment [97]. However, inactivation of some non-enveloped viruses is less [80], and the process appears to be virus-specific.

Various coagulation factors, protease inhibitors, and intravenous IgG are pasteurised during the purification

process. Most often, pasteurisation is performed in the presence of stabilizers, like amino acids, sugars, or citrate [97,98], to protect protein functionality, and limit molecular alterations and protein aggregation. Protein stabilizers are also known to stabilize viruses, however, further highlighting the need for validating the exact conditions of treatment used. Pasteurisation can inactivate viruses of different types, enveloped or non-enveloped, including HIV, HBV, HCV, and HAV [80,96,98,99]. There are few published data on the inactivation of resistant model non-enveloped viruses like porcine parvovirus, SV 40 or Reovirus type 3 in plasma products [100,101]. In most instances, inactivation of such viruses is limited to less than 2–4 log over the 10 h heating period. Heat treatment of whole human plasma at 50 °C for 3 h, in the presence of a specific combination of stabilizers, inactivates more than 6 log of HIV, and more than 4 log of enveloped and non-enveloped model viruses [102]. Finally, it was demonstrated that heat treatments like pasteurisation and vapour heat treatment (as well as solvent-detergent and nanofiltration on 35 nm membranes) can inactivate/remove more than 6 log of WNV [103].

4.2.4. Removal by chromatography

Chromatography is primarily used in human plasma fractionation as a downstream polishing step [104]. Ion-exchange chromatography has been described for some antivenom products [66,67]. Viral partitioning has been shown to occur during affinity and ion-exchange chromatography, but the mechanism of removal is not easy to predict and control [105], making it difficult to consider these as robust viral removal steps.

4.2.5. Storage or formulation at acid pH

Storage at pH 4.25, in the presence of a stabilizer, such as 10% maltose, for a minimum of 21 days at 20 °C, yields aggregate-free and in vitro functionally active human IgG preparations [106]. Incubating at pH 4.25 for 21 days at 20 °C caused a 10,000-fold decrease in BVDV infectivity and complete inactivation of 1000 chimpanzee infectious doses per millilitre of HCV [107]. With the exception of a recent report of a Fab ovine antivenom formulated at pH 4.0 [108], to our knowledge, formulation at low pH is not commonly used for antivenoms but may be considered as a viral safety step, if it does not affect the biological activity, the general safety, and the clinical efficacy of the product.

4.3. Viral validation studies and process implementation

4.3.1. Validation

Viral validation studies are small-scale experiments designed to provide an estimate of the overall viral

reduction level achieved across the manufacturing processes and to identify steps and parameters that are critical for viral inactivation or removal. To perform such studies, as described in the Guidelines prepared by the CPMP, production intermediates are voluntarily spiked with known amounts of known viruses, using laboratory-scale mimicking of the production process, and the virus reduction factor is calculated by comparing the infectivity level before and after each individual process step evaluated. When appropriate, a cumulative viral reduction factor can be calculated and provides information on the viral safety margin of processes for a range of model viruses. Effective and robust individual steps typically achieve more than 4 log reduction in infectivity under a large range of production parameters. Viral validation studies are usually conducted with 3–6 model viruses differing in their structural features (presence of an envelope, RNA or DNA genetic material, size, degree of resistance). “Relevant” viruses, which are known potential contaminant of the starting plasma, should be used when possible. A list of existing laboratory adapted model viruses that could be used for validation studies of antivenoms manufacturing processes is shown in Table 4. VSV and WNV that may contaminate horses appear of special relevance for process validation of equine products.

4.3.2. Process implementation

Dedicated viral inactivation and removal production steps should be implemented in a way to ensure the reproducibility in viral reduction and the absence of risks of downstream contamination. Examples of approaches used for the manufacture of human plasma products are provided in recent WHO guidelines [109], and a CPMP note for guidance provides recommendation on production of immunsera [19]. Both can be used as reference by manufacturers of antivenoms.

Viral reduction equipment should have adapted specifications. For instance, equipment for bulk in

process virus inactivation, such as the acid pH incubation or the caprylic acid treatment, should ideally be fully enclosed. In addition, vessels should have temperature controls and be designed to avoid “dead points” where important parameters such as temperature and pH could not be ensured. Similarly, liquid heat treatment or heat shock should be conducted in jacketed tanks with the solution stirred throughout the heat cycle to ensure homogeneity and all points in the tank should be within the specified temperature range. During the qualification phases, the equipment and the required services should be shown to conform to the predefined technical specifications and requirements, and to function within the specified limits. When possible, it is recommended to create a dedicated “viral safety area” where production steps are arranged in a clear and logical way, so that recontamination of the virally reduced intermediates is avoided. Operating procedures should also be written to reduce the likelihood of cross-contamination. Bulk pH 4 or caprylic acid virus inactivation could be carried out in two stages, the first stage being located in a normal production room, followed by a second incubation in another tank located in a segregated, contained area. The duration of the first stage should be such that the majority of virus inactivation (as found during the viral validation studies) has occurred. On completion of the first stage, the product should be aseptically transferred (e.g. through sterile coupling) into the second vessel located in a safety zone for completion of the second stage of viral inactivation. Product-contacting-equipment used in the safety area should be dedicated or decontaminated to inactivate any remaining virus. A quality assurance system should ensure that the execution of virus reduction methods conforms to the validated conditions. Viral inactivation and removal procedures should be described in approved Standard Operating Procedures (SOPs) that contain critical process limits for the viral inactivation and removal methods applied to antivenoms.

Table 4
Examples of laboratory model viruses that could be used for validation studies of antivenoms

Virus	Family	Envelope	Size (nm)	Genome	Resistance	Model for
Vesicular stomatitis virus	<i>Rhabdoviridae</i>	Yes	50–200	ss-RNA	Low	Relevant virus
West Nile virus	<i>Flaviridae</i>	Yes	40–70	ss-RNA	Low	Relevant virus; model for
Sindbis	<i>Togaviridae</i>		60–70	ss-RNA	Low	Eastern equine encephalitis virus
Bovine viral diarrhoea virus	<i>Togaviridae</i>	Yes	40–60	ss-RNA	Low	Eastern, western and Venezuelan equine encephalitis virus
Parainfluenza	<i>Paramyxoviridae</i>	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	<i>Reoviridae</i>	No	60–80	ds-RNA	Medium	Equine encephalosis virus
Poliovirus; encephalomyocarditis virus; hepatitis A virus	<i>Picornaviridae</i>	No	25–30	ss-RNA	Medium-high	Equine rotavirus

5. Conclusions

To date there is no documented example of viral transmission to humans from the use of antivenoms, whichever type of product (whole IgG, F(ab')₂ or Fab) is manufactured [5]. Considering the difficulty in the containment of large animals like horses, and in an exhaustive viral testing of the starting plasma, the viral safety of antivenoms appears therefore largely dependent upon the capacity of manufacturing processes to reduce infectious agents and on the use of good manufacturing practices, in particular in ensuring production traceability. By contrast to human plasma products, most antivenoms are currently not subjected to purposely introduced viral reduction steps like solvent-detergent, pasteurisation, or nanofiltration [80,109,110]. However, a comparison with validated manufacturing processes used for human IgG clearly indicates that at least two widely used antivenom production steps, caprylic acid treatment and low-pH incubation, are likely to contribute in a robust manner to viral safety, at least against enveloped viruses. The concentration of caprylic acid (5%) used for antivenoms is higher than that used for human IgG (Table 3), and the pH of treatment is within the effective range (less than pH 6) required for optimal inactivation of enveloped viruses. As the treatment is performed on equine plasma itself, or on crude fractions, the viral kill could be reduced by the presence of lipids. The acid incubation of antivenoms which is performed at a pH (3.1–3.8), less than that used for human IgG, and at a similarly warm temperature (30–37 °C) (Table 5) should provide a fast rate of inactivation of lipid-enveloped viruses, and possibly also non-enveloped viruses. However, the incubation time (40 min–24 h) is for some processes significantly less than for human IgG (typically 22–25 h), probably reducing the extent of viral kill achieved. Preliminary viral kill data obtained with antivenoms confirm that conditions used for peptic cleavage at low pH of F(ab')₂ achieve robust inactivation of WNV [111]. We recommend that further representative viral validation studies using a range of well-established virus models are performed to know the rate and extent of viral kill actually achieved during those production steps. The viruses to use for those validations should be discussed with specialized virologists, and, by analogy with most studies done with

human plasma derivatives, might include three lipid-enveloped viruses (such as BVDV, VSV, PSR, and WNV) and one non-enveloped virus (such as EMC, poliovirus, or Reovirus 3). Inactivation data could then be compared to viral kills achieved using similar process steps of human IgG products, therefore providing some reference on their robustness and allowing manufacturers and national regulatory authorities to assess scientifically the margin of safety of antivenom products. Other manufacturing steps, such as heat coagulation, could be validated as well. Formulation of the product at low pH, as done for some human IgG preparations, is another potential approach to consider for improved viral safety, but evaluating product stability and efficacy under such conditions would be a pre-requisite. In view of their expected beneficial impact on viral safety, appropriate process implementation of caprylic acid treatments and low-pH incubations should be ensured, as well as measures taken to avoid risks of downstream contamination and, more widely, to respect good manufacturing practices and traceability concepts. Such conclusions should also be valid for other animal plasma-derived IgG products used in human medicine such as anti-lymphocyte/T-cell, anti-toxins, and anti-bacterial or viral agents sera [19].

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Table 5
Typical conditions for acid pH treatment of human IgG preparations and equine antivenoms

Product	Protein concentration (g/l)	pH	Temperature (°C)	Duration (h)
Human IgG	40–60	4	30–37	20–30
Equine IgG	60–90	3.1–3.8	30–37	0.6–24

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