

Safety of Snake Antivenom Immunoglobulins: Efficacy of Viral Inactivation in a Complete Downstream Process

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Viral safety remains a challenge when processing a plasma-derived product. A variety of pathogens might be present in the starting material, which requires a downstream process capable of broad viral reduction. In this article, we used a wide panel of viruses to assess viral removal/inactivation of our downstream process for Snake Antivenom Immunoglobulin (SAI). First, we screened and excluded equine plasma that cross-reacted with any model virus, a procedure not published before for antivenoms. In addition, we evaluated for the first time the virucidal capacity of phenol applied to SAI products. Among the steps analyzed in the process, phenol addition was the most effective one, followed by heat, caprylic acid, and pepsin. All viruses were fully inactivated only by phenol treatment; heat, the second most effective step, did not inactivate the rotavirus and the adenovirus used. We therefore present a SAI downstream method that is cost-effective and eliminates viruses to the extent required by WHO for a safe product. © 2013 American Institute of Chemical Engineers Biotechnol. Prog., 29:972–979, 2013

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Introduction

Humankind has suffered from poisoning because of animal bites since the beginning of times. Currently, snakebites represent the main cause of human envenoming, with approximately 1,841,000 occurrences and 94,000 deaths yearly. Most of the occurrences happen in Asia, Latin America, and especially in Africa.¹

Snake Antivenom Immunoglobulins (SAI) may prevent the referred morbidity and mortality if the correct antidote is administered immediately after the bite. These conditions, however, are impracticable for most regions in need and for some species of snake.^{1,2} The World Health Organization (WHO) endorsed the relevance of SAI for global health by including them in the “Model Lists of Essential Medicines.”³

All biological health products require a rigorous quality control, including SAI. The WHO recently compiled the quality parameters for this centenary product in “WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins.” The document emphasizes that viral content is an essential control parameter because contaminated SAI may infect humans and lead to morbidity or even mortality. Indeed, contaminated human-derived immunoglobulin led to several cases of human

infections with hepatitis C virus.⁴ The phenomenon is so far unreported for animal-derived immunoglobulins.^{5,6}

SAI are purified from hyperimmunized plasma of animals, mainly horses, immunized with a pre-determined amount of crude venom from one or more snake species.^{2,7} Ideally, the donor animals should be free of viral pathogens,⁸ but this approach fails because of the incomplete understanding of the equine virology and consequent lack of vaccines.⁹ Therefore, to compensate for this flaw, viral inactivation becomes essential in the downstream process of equine plasma.

SAI purification diminishes viral load of contaminated plasma, although only process validation guarantees full elimination.^{9,10} Some researchers reported viral elimination throughout SAI purification,^{11,12} but none described a prior plasma selection based on antiviral titers. This selection excludes plasma samples that are positive for antibodies against the model viruses used for validation. Without selection, neutralizing antibodies may interfere in the measurement of virus titers,¹³ an essential parameter to evaluate the capability of virus removal/inactivation of the steps alone. Whenever a whole process fails to complete viral inactivation, modifications like further steps might improve the outcome. Some preservatives inactivate viruses cost-effectively, representing a plausible alternative to eliminate viral activity. For example, SAI formulations commonly contain phenol⁷ that, although known as virucidal, has not been explored to this end for SAI production in the literature.

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Table 1. Virus Strains for Modeling Viral Inactivation

Virus Model	Family	Envelope Presence*	Genetic Material	Represented Viruses†,‡
Bovine Herpesvirus-1 (BHV-1)	Herpesviridae	+	dsDNA	EHV†, EPV, BPV
Canine Adenovirus-1 (CAV-1)	Adenoviridae	–	dsDNA	EAdV-1
Canine Parvovirus (CP)	Parvoviridae	–	ssDNA	EP
Bovine rotavirus (BRV)	Reoviridae	–	dsRNA	ERAV, ERBV, AHS, EEV, ER, RV1-3†
Canine Distemper Virus (CDV)	Paramyxoviridae	+	(–) ssRNA	BV†, IAV†, Hendra†, Nipah†
Vesicular Stomatitis Virus (VSV)	Rabdoviridae	+	(–) ssRNA	VSV†, BV†, IAV†
Canine Coronavirus (CCV)	Coronaviridae	+	(+) ssRNA	EC, EAV, EEEV†, WEEV†, VEEV†, EAV
Bovine Viral Diarrhea Virus (BVDV)	Flaviviridae	+	(+) ssRNA	WNFV†, JBEV†, SLEV†

*Present (+) or absent (–).

†Virus classified as pathogenic to horses and humans.

‡AHS, african horse sickness; BPV, bovine papilloma virus; BV, borna virus; EAdV, equine adenovirus type 1; EAV, equine arteritis virus; EC, equine coronavirus; EEEV, eastern equine encephalitis virus; EEV, equine encephalosis virus; EHV, equine herpes virus; EP, equine parvovirus; EPV, equine papillomavirus; ER, equine rotavirus; ERAV, equine rhinitis A virus; ERBV, equine rhinitis B virus; Hendra, equine morbilli virus; IAV, influenza A virus; JEV, japanese B encephalitis virus; RV1-3, reovirus 1-3; SLEV, Saint Louis encephalitis virus; VEEV, venezuelan equine encephalitis virus; VSV, vesicular stomatitis virus; WEEV, western equine encephalitis virus; WNV, west Nile fever virus.

To address the above issues, we classified and selected plasma from donor horses (Instituto Butantan, Brazil) according to their antiviral immunoglobulin titers. After selection, we assess viral safety of the SAI process developed by Instituto Butantan and evaluate the efficacy of phenol as a virucidal agent for SAI.

Material and methods

Immunized equine plasma

We chose the venom of *Crotalus durissus terrificus* (South American rattlesnake, cascavel) as a model for immunization. The choice was random because our institute produces several snake antivenoms from equine plasma with the same procedure. The type of venom does not influence the outcome of virus removal.

Fifteen healthy adult horses were immunized by intramuscular route against the snake venom. These animals were tested for Equine Infectious Anemia Virus every 6 months and were considered free from this virus. All animals were also previously immunized against rabies, equine influenza virus, leptospirosis, tetanus, and Venezuelan equine encephalitis virus. The immunization scheme to produce antivenom serum consisted in three inoculation days (0, 14, and 21). On each inoculation day, 10 injections of 0.5 mL venom solution were applied to different spots within the lumbar region. Blood collection was done by the jugular vein from each horse (maximum of 4% horse weight), 2 weeks after immunization. Plasma was obtained by gravity sedimentation of blood (18 h/5–8°C) and tested for sterility/potency, mycoplasma content (Mycoplasma PCR ELISA, Roche applied Science), and virus screening. After plasma isolation, blood cells were returned to the donor horses (plasmapheresis).

Day 0—Crotalus venom (2 mg/mL, Instituto Butantan, Brazil) mixed with the adjuvant Montanide ISA50 25% (mineral oil emulsion, Seppic, Brazil); day 14—Crotalus venom (2 mg/mL) diluted in saline solution; and day 21—Crotalus venom (4 mg/mL) diluted in saline solution.

Virus strains and cell lines

The viruses chosen as models for viral inactivation studies are described in Table 1. To replicate these viruses and test their cytopathic effects, five different cell lines were used (Table 2). All cell lines were free of mycoplasma (Mycoplasma PCR ELISA, Roche applied Science).

Cell culture

All cells were cultivated as monolayers in cell culture flasks with Dulbecco Modified Eagle’s Medium (DMEM, Sigma-Aldrich), supplemented with 10% Fetal Bovine Serum—FBS (Cutlab, Brazil), and incubated at 37°C, 5% CO₂. For subcultivation, passages were performed when a cell monolayer reached 80% confluence (ratio of 1:3–1:8). Cell detachment for sub cultivation was achieved by addition of 0.25% trypsin (Sigma Aldrich) to a 0.02% EDTA solution (Merck).

Virus propagation

Each virus was replicated using the appropriate cell line (Table 2), while the cell culture was performed as described (item 2.3). Cell culture monolayer at 80% confluence was infected with virus (multiplicity of infection of 1:1,000) and incubated again. The supernatant was collected after identification of visual cytopathic effect in the monolayer. Cell debris were removed by centrifugation (30 min/4°C/3000× g) and the remaining supernatant containing the replicated virus was titrated and stored in –70°C

Table 2. Virus Strains and Respective Cell Lines

Virus	Cell Lines (Amplification)*	Cell Lines (TCID 50%/MTT)†
BHV-1 (ATCC VR188)	MDBK (ATCC CCL-22)	MDBK
CAV-1 (ATCC VR293)	CRFK (ATCC CCL 94)	MDCK
CP (ATCC 2017)	CRFK	MDCK (CCL-34)
BRV (ATCC VR1290)	VERO (ATCC CCL-81)	MDBK
CDV (ATCC VR1587)	VERO	VERO
VSV (ATCC VR158)	VERO	BHK (ATCC CCL-10)
CCV (ATCC VR2068)	CRFK	MDCK
BVDV(ATCC VR1462)	MDBK	MDBK

*Cell lines used for virus amplification.

†Cell lines used for the TCID50% test (item 2.5) and MTT test (item 2.8).

freezer. Virus storage was performed at a minimum concentration of 10^6 log measured by TCID₅₀. To achieve the minimum concentration for storage, Canine Distemper Virus and Canine Adenovirus were treated with PEG 6,000 (8% v/v) for 18 h/4°C followed by centrifugation (30 min/4°C/5,000× g), supernatant discharge and pellet resuspension with DMEM (0.5 mL/100 mL initial plasma volume).

Virus titration by tissue cytopathic infectious dose 50%

Cells for TCID₅₀ test (Table 2) were cultivated (item 2.3) and seeded overnight in 96-well cell culture plates (1 plate per virus, 100 μL containing 20,000 cells per well). Ten-fold serial dilutions of each virus sample were prepared and inoculated (100 μL) into the wells of the respective plates, with eight replicates per dilution. A row with culture medium was used as the negative infection control. The infected plates were incubated for 1 h for virus infection in DMEM 5% FBS (37°C/5% CO₂), followed by medium renewal (DMEM 5% FBS) and new incubation until maximum cytopathic effect (up to 5 days). After incubation, cells were fixed with 7% formaldehyde and stained with 5% crystal violet. The infected wells did not stain, whereas the wells containing intact cells stain blue/purple. For each virus experiment, the average of three virus assays were used for TCID₅₀, which was calculated by the Spierman–Karber method.¹⁴

Antibody screening against viruses by ELISA

The 15 samples from equine serum immunized against *Crotalus durissus terrificus* were screened for eight different viruses (Table 1) by direct enzyme-linked immunosorbent assay (ELISA).¹⁵ Unless otherwise stated, all incubations were performed at 37°C, with washing steps (PBS/Tween 0.25%) between incubations. For each virus strain, a 96-well plate (cat. no. 3590, Corning) was coated with 100 μL of virus solution (10 μg/mL protein content), diluted in carbonate buffer (0.1 M, pH 9.6), and incubated overnight at 4°C. Following a 2 h blocking incubation step (0.5% gelatin), 100 μL of serum samples serially diluted (10-fold) were added to the plate and further incubated for 2 h. For serum IgG detection, rabbit antihorse IgG conjugated with peroxidase (Sigma, EUA) was incubated for 1 h (100 μL/well, 10,000 times diluted). A further incubation at room temperature was then performed with the substrate *O*-phenylenediamine (Sigma, EUA) and hydrogen peroxide (Nuclear, Brazil) 0.03%. The reaction was stopped after 15 min with sulfuric acid 2.5 N. The plate was read at 492 nm (Titertech® Spectronic). Serum samples that presented an optical density of ≤0.100 (background) for all dilutions were considered free of antibody to the tested viruses. All tested samples were assayed in triplicate and repeated for confirmation.

SAI purification process

The SAI purification process was developed in Instituto Butantan, Brazil, to obtain crotalic antivenom derived from equine plasma. This process is an optimization of a previously developed method.^{16–18} The flowchart (Figure 1) describes the process for obtaining the concentrated immunoglobulin solution, in which the steps identified with potential for viral inactivation are highlighted. The method and tests were already described in item 2.1. When a routine batch is produced, the concentrated bulk is further diluted, filter sterilized, and packaged.

Cytotoxic evaluation of viral inactivation treatments

Cells (Table 2) for cytotoxicity were cultivated (item 2.3) and seeded overnight in 96-well plates for cell culture (20,000 cells per well). The test samples were taken immediately after each of the four viral inactivation steps (item 2.7) and diluted serially (twofold) in culture medium. Diluted samples were loaded to the plate for incubation (24 h/37°C/5% CO₂). Following cell culture medium removal, 50 μL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, EUA] solution was added and further incubation was performed for 2 h. After MTT removal, 100 μL of isopropanol was used to dissolve MTT crystals and the resulting optical density was measured at 590 nm (Titertech® Spectronic). The average optical density of control wells that did not receive virus represented 100% viability. All other wells had their viability calculation based on these controls.

Viral removal assessment

The capability of viral inactivation was evaluated for the chosen steps following directions from the guidelines and papers published before.^{19,20} The Q5A guideline is also related to viral removal/inactivation validation, although not designed for animal plasma derivatives.²¹

A purification process without any spiking was performed as a blank. The process (Figure 1) was conducted routinely until before the chosen step, when the sample was spiked with one model virus. After that, the step was performed as described and the resultant product evaluated by TCID₅₀. Each model virus was evaluated in a separate experiment for each step. Immediately after the spiking and before the evaluated step, duplicate samples were collected and stored (–70°C) for quantification of initial TCID₅₀ at the same time as the final TCID₅₀. All test samples were stored (–70°C) until the analyses. The spiking rate used was 1:50 for all runs, and the TCID₅₀ of nonspiked runs were considered the test blank. The viral log reduction factor (LRF) was then calculated for each run, as described below:

$$\text{Log reduction factor (LRF)} = \log(V1 \times T1) / (V2 \times T2) \quad (1)$$

V1 = volume of starting material,
T1 = concentration of virus in starting material (initial TCID₅₀),
V2 = volume of material after the evaluated step,
T2 = concentration of virus after the step.

Results and Discussion

Antiviral immunoglobulin screening of horse-derived plasma samples

In order to verify virus elimination in equine plasma, we chose eight strains of viruses as models of all significant viruses reported to infect horses (Item 2.2, Table 1).⁸ We then screened plasma samples from donor horses for antiviral immunoglobulins, detected by ELISA. The screening showed 9 positive samples out of 13, considering positive a sample that recognized at least one model virus. Viral recognition was independent of the type of genetic material or presence of the envelope, but related with viral origin (Figure 2), with the majority of samples reacting with bovine viruses, especially the BHV-1 and BVDV, followed by canine viruses. Because the cited viruses generally do not infect horses, the result probably arose from cross-reactivity of the antibodies.

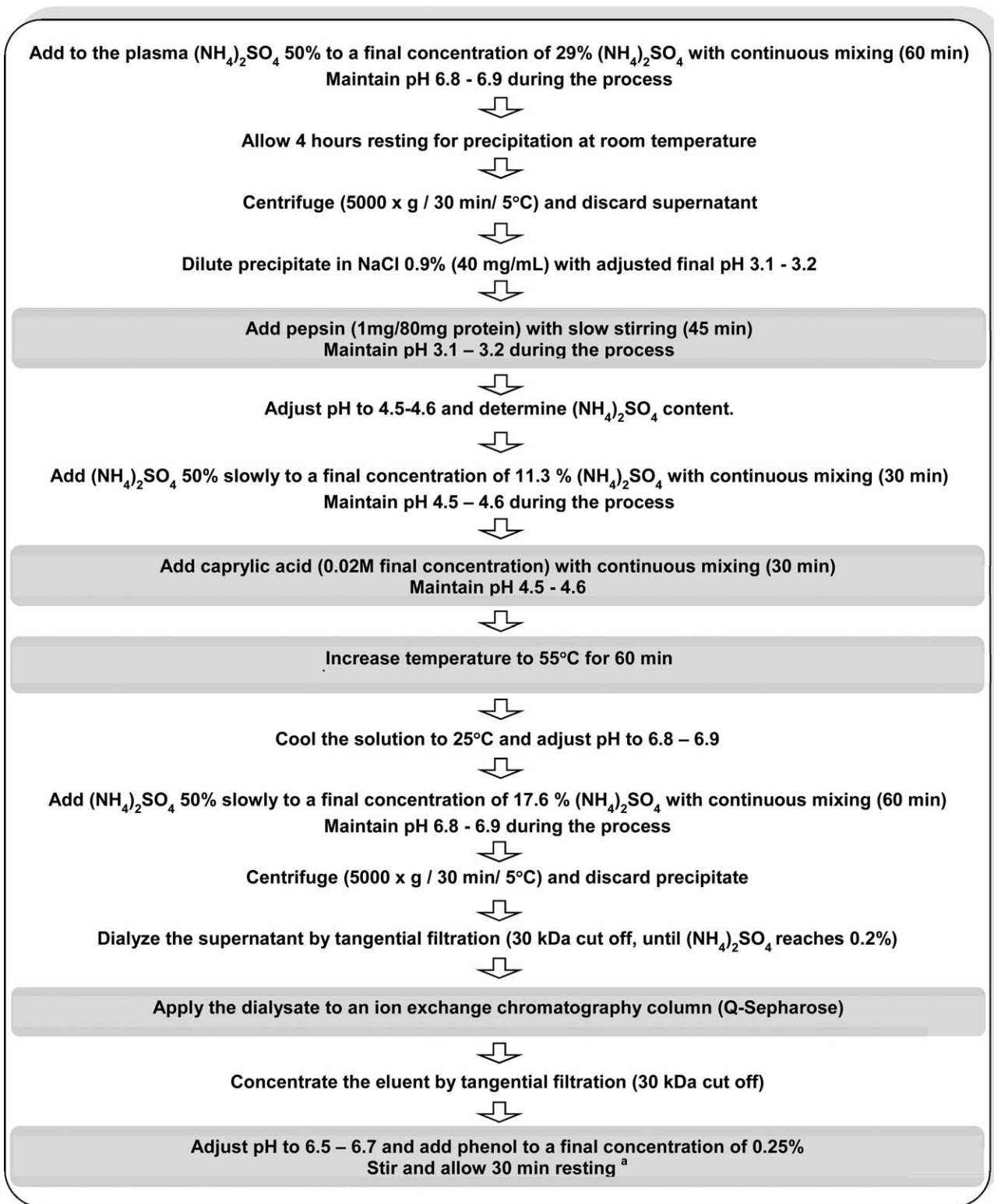


Figure 1. Process flowchart to obtain purified SAI. (a) The bulk product is further filter sterilized and held for at least 21 days (quality control testing period). After the formulation (0.15% phenol concentration), the final product is packed (ampoules).

Antibody cross-reactivity may occur with viruses that infect several species of animals,^{22,23} but no reports broached the subject with bovine or canine and equine viruses.

Two plasma samples had antibodies against vesicular stomatitis virus (VSV), the only model virus in this work that

commonly infects horses (Figure 2). However, all animals were free of VSV (PCR screening, data not shown), leaving previous infection or antibody cross-reactivity as probable causes for positives. Researchers found that crude plasma does contain a mixture of immunoglobulins that cross-reacts

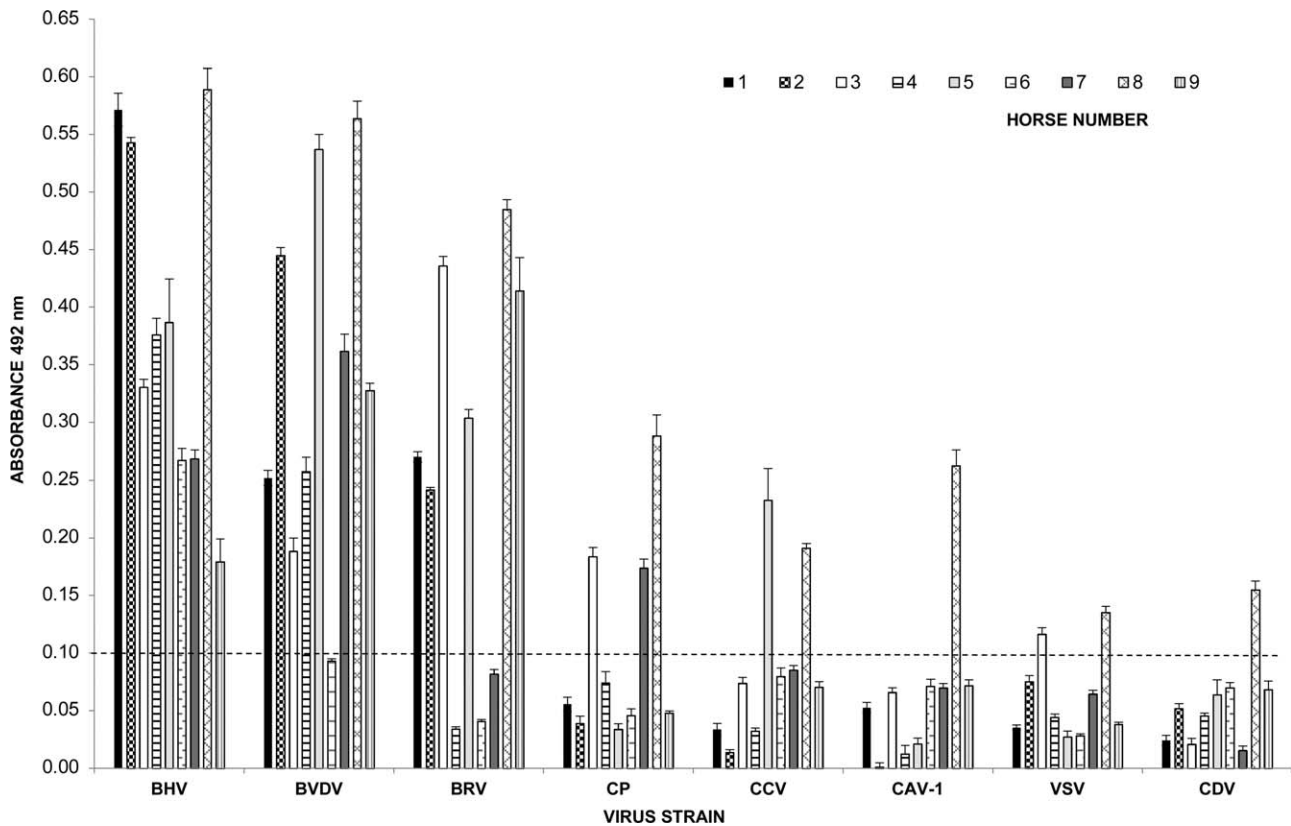


Figure 2. Antibody screening against model viruses presented in plasma samples. Thirteen horse samples were screened by direct ELISA (item 2.6); four were negative for all viruses (not shown) and nine were positive, represented in the above graphic. Bars represent the mean of triplicate samples and error bars represent the standard deviation. The dotted line highlights the value 0.10, considered the background of the test.

with various antigens, supporting our results.²⁴ The positive samples were excluded from further inactivation studies to allow the specific viral elimination step to be tested.

Potency of SAI is defined as the amount of snake venom neutralized by 1 mL of plasma (mg/mL), measured in this work as described before.²⁵ The four negative samples neutralized at least 1 mg/mL, the minimum potency required for further purification (data not shown). The pool from these samples also surpassed the minimum protein value (1.61 mg/mL) and constituted the starting material for validation studies.

Assessment of viral inactivation of purification steps

The process to obtain purified SAI is described in detail in the methods section (item 2.7, Figure 1). The main steps are cited in sequential order (a) ammonium sulfate precipitation of immunoglobulins, (b) pepsin digestion to obtain F(ab')₂ fragments, (c) caprylic acid precipitation of nonimmunoglobulin proteins, (d) heat treatment, (e) ammonium sulfate precipitation of F(ab')₂ fragments, (f) tangential filtration, (g) ion-exchange chromatography, (h) tangential filtration, and (i) phenol addition.

Among all procedures, we chose (b), (c), (d), and (i) for viral inactivation studies because of their reported virucidal capability.^{8,11,26,27} Ion-exchange chromatography (g) also removes viruses,^{28,29} but evaluation of this step would require sanitization used between batches. Thus, we performed several purification processes of the pooled sample, each one spiked before step (b), (c), (d), and (i) with the eight model viruses individually (item 2.9). The spiking concentrations (Table 3) were different for each virus, a

consequence of the replication yield obtained: CDV and CAV-1 titers were consistently below six logs and required concentration before spiking, while BRV titer decreased slightly above six logs after mixing (5.4 log) (Table 3).

We analyzed cell cytotoxicity of downstream steps before the spiking experiments. Samples taken before and after each chosen step were toxic up to 12.5% (diluted in culture medium). Therefore, we used 6.25% dilution for cell-based assays. There were no cytotoxicity differences between samples.

To assure viral safety, spiking experiments with initial virus titers of six logs should result in undetectable virus load. However, the process still contributes to viral safety if the reduction is between four and six logs, but requires additional viral reduction to meet European Agency for the Evaluation of Medicinal Products (EMA) requirements.³⁰ By contrast, a process step presenting LRFs below four logs generally inactivates an insufficient amount of virus to insure adequate safety.³¹

We calculated viral LRFs after each downstream step, and all of them were at the least 1.0 log (Table 3), which is considered the threshold.³⁰ The effectiveness of the steps was analyzed by the amount of viruses inactivated that resulted in LRFs greater than 6 logs (effective), 4–6 logs (contribute to viral safety), or below 4 logs (ineffective). The collected data pointed out heat treatment as more effective than pepsin or caprylic acid. Nevertheless, heat treatment was performed in the presence of residual caprylate from the previous step, which may have influenced LRF values (2–7 log). All LRFs were about 3–7 log and 2–6 log for the pepsin and caprylic process steps, respectively, depending on the viruses

Table 3. Viral LRF Obtained in Each Evaluated Step of SAI Production

Virus	Envelope Presence*	Genetic Material	LRF [†] Pepsin	LRF [†] Caprylic acid	LRF [†] Heat [‡]	LRF [†] Phenol [§]	Cumulative LRF**
BHV-1	+	dsDNA	6.90 ± 0.11	5.96 ± 0.10	7.08 ± 0.14	≥8.11	≥28.05
CCV	+	(+) ssRNA	5.95 ± 0.08	6.03 ± 0.06	6.80 ± 0.10	≥10.13	≥28.91
VSV	+	(-) ssRNA	5.75 ± 0.08	4.83 ± 0.07	6.49 ± 0.13	≥8.99	≥26.06
BVDV	+	(+) ssRNA	5.84 ± 0.14	4.33 ± 0.14	6.44 ± 0.14	≥7.58	≥24.19
CP	-	ssDNA	4.30 ± 0.20	5.18 ± 0.16	5.30 ± 0.18	≥6.30	≥21.08
CDV	+	(-) ssRNA	3.99 ± 0.07	4.07 ± 0.05	4.94 ± 0.06	≥8.12	≥21.12
CAV-1	-	dsDNA	3.10 ± 0.08	3.18 ± 0.06	4.11 ± 0.09	≥7.27	≥17.66
BRV	-	dsRNA	2.69 ± 0.11	1.52 ± 0.09	2.27 ± 0.11	≥5.41	≥11.89

VSV, vesicular stomatitis virus; BHV-1, bovine herpesvirus-1; CCV, canine coronavirus; BVDV, bovine viral diarrhea virus CP; CDV, canine distemper virus; CAV-1 canine adenovirus-1; BRV, bovine rotavirus.

*Present (+) or absent (-).

[†]Arithmetic mean values from triplicate measurements, followed by confidence interval (95%).

[‡]Heat step was performed in the presence of residual caprylate from previous step.

[§]Phenol treatment resulted in undetectable viral loads, reason why the LRF values are the same as the initial titers.

**The sum of LRFs for all treatments, calculated for each virus type.

involved. Just the phenol addition step resulted in undetectable viral load.

Differences in virus susceptibility to pepsin, caprylic acid, and heat treatments

Enveloped Viruses. In our experiments, the most susceptible viruses were BHV-1 and CCV, with LRFs ≥ 6 logs after each treatment. BHV-1, as other herpesviruses, generally presents low resistance to antiviral treatments,^{32,33} whereas heat (56–65°C, 30 min⁻¹/h) and low pH might reduce CCV infectivity.³⁴ We found no reports using both viruses as models for viral inactivation concerning the described purification steps.

VSV and BVDV generally resists to inactivation more than most enveloped viruses,^{11,35} but are extensively neutralized with heat treatment.^{36,37} Indeed, we observed LRF values of BVDV and VSV above six logs when treated with phenol or when heated, but between six and four logs when treated with pepsin or with caprylic acid (Table 3).

In respect to VSV, other researchers also found LRFs in the same range as we did for pepsin and caprylic acid treatments.^{32,38} Another experiment showed that both treatments reduced approximately 3.5 logs of VSV after 20 min, followed by consisted inactivation (>6 logs) after 60 min.¹¹ The latter finding suggests that inactivation could be extended for VSV if our process reached 1 h instead of 45 min for pepsin and 30 min for caprylic acid. Nevertheless, we have to consider that extending these parameters may also diminish serum potency.³⁹ Because our protocol achieved an extended inactivation in the heat step, as reported before,⁴⁰ it is unnecessary to extend steps that might decrease activity to kill this specific virus.

Concerning BVDV, previous literature showed a similar trend to our results with caprylic acid and pepsin treatment at pH 4,³⁶ except for a work reporting high-BVDV resistance with pepsin treatment at pH 3.¹¹ The higher resistance found in that work might be because of spiked plasma containing BVDV antibodies. If the plasma contained antibodies bound to BVDV, the initial titer may have been underestimated. The decrease in pH might have destabilized the previous antibody-antigen binding¹³ and released infective viruses that were not detected at the beginning. The consequence of these events is an underestimated reduction factor.

The four enveloped viruses discussed above were inactivated in a greater extent than nonenveloped viruses, to the known general properties of viruses.^{41,42} An exception to the

trend was the enveloped CDV, which presented LRFs ≥ 6 only with phenol addition. This resistance probably arose from animal protein/virus interaction because previous reports described CPV protection from inactivation when calf serum or egg tissue was present.^{43,44} The later finding is quite relevant, illustrating that viruses easily inactivated in the environment can be quite resistant in biological fluids and therefore should be included in validation panels.

Nonenveloped Viruses. The most resistant virus detected in our experiment was BRV-1, poorly neutralized by pepsin, caprylic acid, and heat treatments (Table 3). BRV was also poorly neutralized with acidic treatment (pH 3–4, 60 min).⁴⁵

CAV-1 was not effectively inactivated by pepsin and caprylic steps (LRF <4), but it was moderately inactivated after the heat step at acid pH (6–4 log) in our experiments. Yamamoto and colleagues inactivated more logs of CAV-1 with increased pepsin concentration and extensive reaction times, up to 4 h.⁴⁶ However, these increments can cause loss of activity, as discussed before. In another paper, Petr and Jiran completely inactivated CAV-1 in serum-free medium, pH 7.2, after heat inactivation.⁴⁷ This increased sensibility to heat probably results from the pH used because CAV-1 is particularly unstable at neutral pH range.⁴⁸

Caprylic and pepsin treatments are generally considered unsuitable for inactivation of nonenveloped viruses.³⁶ Again, we found an exception to an established trend because the nonenveloped CPV was substantially inactivated after the referred treatments (6–4 log). We could not find papers using canine parvovirus for validation of the referred treatments in crude plasma samples. We will avoid performance comparison with other parvoviruses or different initial products because of high variability found among the parvovirus group.^{36,49,50} Nevertheless, CPV seems to be quite vulnerable to acidic pH, the same condition maintained in our pepsin step.⁵¹ In order to guarantee the “worst-case scenario” for parvovirus inactivation, one would have to test different parvoviruses in the same conditions as we tested CPV.

Phenol as a virucidal preservative

Phenol is a common preservative for products derived from animal immunoglobulins. Its efficacy against bacteria has been extensively studied, but reports approaching phenol as virucidal are scarce in the literature. Addition of phenol to the concentrated bulk resulted in inactivation of all model viruses after just 30 min of exposure, evidenced by undetected values of TCID 50%. A partial or total inactivation was expected for

HBV-1, CV, VSV, BVDB, and CDV, because phenolic compounds were described as efficient virucidal agents of enveloped viruses. However, inactivation by phenol of nonenveloped viruses differs with the virus species studied.⁵²

Some model viruses used in this work are also models for human viruses. For instance, BVDV is used as a surrogate model of hepatitis C virus⁵³ and CPV as a model of human parvovirus B19.⁵⁴ These viruses are important agents of viral infection through human blood products and, consequently, targets for viral inactivation.^{52,55} Phenol inactivated both viruses, which indicates that it might also be an effective preservative for human-derived immunoglobulins, when it comes to viral safety.

Concerning phenol safety, its use in high concentrations ($\geq 0.25\%$) was reported to facilitate formation of IgG aggregates and dimmers, contributing to turbidity of the solutions at 4°C. *In vivo* effects in mice include transient hypotension and disturbance of leukocyte–endothelial interactions. Diluted phenol was not reported as toxic or as a destabilizing agent of the formula.²⁷ The process described here utilizes 0.25% phenol in the concentrated bulk, but after 30 min the batch product is diluted, which abrogates the possibility of the reported side effects.

Conclusion

Antibody cross-reactivity is a common feature of viruses of the same species or genus that infect different animals. We found several evidences of virus inactivation and thus of possible cross-reaction, showing for the first time that equine plasma recognizes a wide array of typical model viruses, including a substantial amount of BVDV. Because this virus commonly belongs to validation panels, especially for HCV modeling, the viral screening becomes essential before evaluating any virucidal step.

Concerning the performance of the steps, phenol was the most effective one, followed by heat, caprylic acid, and pepsin. This was an expected result because phenol and heat were the only treatments dedicated to eliminate microbes.

Ideally, phenol should not be used as a complement for bactericidal purposes, but its virucidal properties are quite important when resistant nonenveloped viruses, poorly inactivated by other treatments, are present.⁷ That was the case of this protocol because only phenol was able to inactivate all viruses. Phenolic compounds were additionally reported as virucidal for equine infectious anemia virus, an essential feature when donor horses are located in endemic regions for this enveloped virus.⁵⁶ Other processes might remove the resistant viruses, like nanofiltration,³⁶ but the production costs surpass the budget of the regions in need.

We therefore present a SAI purification method that is cost-effective and eliminates viruses to the extent required by WHO for a safe product. In addition, our process may be applied to any equine-derived immunoglobulin, like spider/scorpion antivenoms or rabies immunoglobulin. Their production variables (antigen obtention and horse immunization scheme) happen before plasma collection and therefore do not interfere with the downstream process.

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Literature Cited

- Kasturiratne A, Wickremasinghe AR, De Silva N, Gunawardena NK, Pathmeswaran A, Premaratna R, Savioli L, Laloo DG, de Silva HJ. The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths. *PLoS Med.* 2008;5:e218.
- Espino Solis GP, Riaño-Umbarila L, Becerril B, Possani LD. Antidotes against venomous animals: state of the art and perspectives. *J Proteomics.* 2009;72:183–199.
- WHO. WHO | WHO model lists of essential medicines. WHO. Available at: <http://www.who.int/medicines/publications/essentialmedicines/en/>. Accessed July 11, 2012.
- Razvi S, Schneider L, Jonas MM, Cunningham-Rundles C. Outcome of intravenous immunoglobulin-transmitted hepatitis C virus infection in primary immunodeficiency. *Clin Immunol.* 2001;101:284–288.
- Quinti I, Pierdominici M, Marziali M, Giovannetti A, Donnanno S, Chapel H, Bjorkander J, Aiuti F. European surveillance of immunoglobulin safety—results of initial survey of 1243 patients with primary immunodeficiencies in 16 countries. *Clin Immunol.* 2002;104:231–236.
- Carbone J, Sarmiento E, Fernandez Cruz E. Overwhelming course of HCV disease in patients with hypogammaglobulinemia associated with cellular immunity deficiency. *Rev Gastroenterol Peru.* 2005;25:19–22.
- Gutierrez JM, Leon G, Burnouf T. Antivenoms for the treatment of snakebite envenomings: the road ahead. *Biologicals.* 2011;39:129–142.
- Burnouf T, Griffiths E, Padilla A, Seddik S, Stephano MA, Gutierrez JM. Assessment of the viral safety of antivenoms fractionated from equine plasma. *Biologicals.* 2004;32:115–128.
- Theakston RDG, Warrell DA, Griffiths E. Report of a WHO workshop on the standardization and control of antivenoms. *Toxicol.* 2003;41:541–557.
- Rossi F, Legras JF. Viral safety: european and french directives. *Ann Med Interne (Paris).* 2000;151(Suppl 1):1S55–1S61.
- Burnouf T, Terpstra F, Habib G, Seddik S. Assessment of viral inactivation during pH 3.3 pepsin digestion and caprylic acid treatment of antivenoms. *Biologicals.* 2007;35:329–334.
- Solano S, Segura Á, León G, Gutiérrez J-M, Burnouf T. Low pH formulation of whole IgG antivenom: impact on quality, safety, neutralizing potency and viral inactivation. *Biologicals.* 2012;40:129–133.
- Petschow BW, Talbott RD. Reduction in virus-neutralizing activity of a bovine colostrum immunoglobulin concentrate by gastric acid and digestive enzymes. *J Pediatr Gastroenterol Nutr.* 1994;19:228–235.
- Hamilton M, Russo R, Thurston R. Trimmed Spearman-Kärber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol.* 1977;11:714–719.
- Engvall E. Enzyme immunoassay ELISA and EMIT. *Meth Enzymol.* 1980;70:419–439.
- Pope CG. The action of proteolytic enzymes on the antitoxins and proteins in immune sera: I. True digestion of the proteins. *Br J Exp Pathol.* 1939;20:132–149.
- Pope CG. The action of proteolytic enzymes on the antitoxins and proteins in immune sera: II. Heat denaturation after partial enzyme action. *Br J Exp Pathol.* 1939;20:201–212.
- Pope CG, Stevens MF. The action of proteolytic enzymes on the antitoxins and proteins in immune sera. *Br J Exp Pathol.* 1951;32:314–324.
- Larzul D. Viral validation design of a manufacturing process. *Dev Biol Stand.* 1999;99:139–150.
- WHO. WHO | WHO guidelines for the production, control and regulation of snake antivenom immunoglobulins. WHO. Available at: http://www.who.int/bloodproducts/snake_antivenoms/snakeantivenomguide/en/. Accessed July 11, 2012.
- Guideline, ICH Harmonized Tripartite. Q5A: quality of biotechnological products: viral safety evaluation of biotechnology

- products derived from cell lines of human or animal origin. *Fed Reg.* 1998; 63:51074.
22. Nagai M, Ito T, Sugita S, Genno A, Takeuchi K, Ozawa T, Sakoda Y, Nishimori T, Takamura K, Akashi H. Genomic and serological diversity of bovine viral diarrhoea virus in Japan. *Arch Virol.* 2001;146:685–696.
 23. Wellenberg GJ, Mars MH, Van Oirschot JT. Antibodies against bovine herpesvirus (BHV) 5 may be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA. *Vet Microbiol.* 2001;78:79–84.
 24. Guidry AJ, O'Brien CN. A method for measuring specific antibodies in bovine lacteal secretions during the nonlactating period. *J Dairy Sci.* 1996;79:846–850.
 25. Campos ACMR, Freitas MCWD, Yamaguchi IK, Marcelino JR, Dias da Silva W. A sensitive and specific immunoassay for the measurement of the antibodies present in horse antivenoms endowed with the capacity to block the phospholipase a₂-dependent hemolysis induced by snake venoms. *J Venom Anim Toxins.* 1996;2:106–120.
 26. Nowak T, Gregersen J-P, Klockmann U, Cummins LB, Hilfenhaus J. Virus safety of human immunoglobulins: efficient inactivation of hepatitis C and other human pathogenic viruses by the manufacturing procedure. *J Med Virol.* 1992;36:209–216.
 27. Rojas G, Vargas M, Robles A, Gutiérrez JM. Turbidity of hyperimmune equine antivenom: the role of phenol and serum lipoproteins. *Toxicon.* 1993;31:61–66.
 28. Buchacher A, Iberer G. Purification of intravenous immunoglobulin G from human plasma—aspects of yield and virus safety. *Biotechnol J.* 2006;1:148–163.
 29. Biescas H, Gensana M, Fernández J, Ristol P, Massot M, Watson E, Vericat, F. Characterization and viral safety validation study of a pasteurized therapeutic concentrate of antithrombin III obtained through affinity chromatography. *Haematologica.* 1998;83:305–311.
 30. CPMP. Note for guidance on production and quality control of animal immunoglobulins and immunera for human use. European Agency for the Evaluation of Medicinal Products (EMA). Available at: <http://www.who.int/bloodproducts/publications/EMA-animal%20sera.pdf>. Accessed July 30, 2012.
 31. WHO. WHO | Technical report, Series No. 924, 2004, annex 4: guidelines on viral inactivation and removal procedures intended to assure the viral safety of human blood plasma products. WHO. Available at: http://www.who.int/bloodproducts/publications/WHO_TRS_924_A4.pdf. Accessed July 11, 2012.
 32. Henzler H-J, Kaiser K. Avoiding viral contamination in biotechnological and pharmaceutical processes. *Nat Biotechnol.* 1998; 16:1077–1079.
 33. Kempf C, Jentsch P, Poirier B, Barré-Sinoussi F, Morgenthaler JJ, Morell A, Germann D. Virus inactivation during production of intravenous immunoglobulin. *Transfusion.* 1991;31:423–427.
 34. Pratelli A. Canine coronavirus inactivation with physical and chemical agents. *Vet J.* 2008;177:71–79.
 35. Lundblad JL, Seng RL. Inactivation of lipid-enveloped viruses in proteins by caprylate. *Vox Sang.* 1991;60:75–81.
 36. Caballero S, Nieto S, Gajardo R, Jorquera JJ. Viral safety characteristics of Flebogamma® DIF, a new pasteurized, solvent-detergent treated and Planova 20 nm nanofiltered intravenous immunoglobulin. *Biologicals.* 2010;38:486–493.
 37. Mpandi M, Schmutz P, Legrand E, Duc R, Geinoz J, Henzelin Nkubana C, Giorgia S, Clerc O, Genoud D, Weber T. Partitioning and inactivation of viruses by the caprylic acid precipitation followed by a terminal pasteurization in the manufacturing process of horse immunoglobulins. *Biologicals.* 2007;35:335–341.
 38. Korneyeva M, Hotta J, Lebing W, Rosenthal RS, Franks L, Petteway SR Jr. Enveloped virus inactivation by caprylate: a robust alternative to solvent-detergent treatment in plasma derived intermediates. *Biologicals.* 2002;30:153–162.
 39. Morais V, Massaldi H. Effect of pepsin digestion on the antivenom activity of equine immunoglobulins. *Toxicon.* 2005;46:876–882.
 40. Uemura Y, Yang YH, Heldebrandt CM, Takechi K, Yokoyama K. Inactivation and elimination of viruses during preparation of human intravenous immunoglobulin. *Vox Sang.* 1994;67:246–254.
 41. Maillard J-Y. Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems. *Ther Clin Risk Manag.* 2005;1:307–320.
 42. Moore MA. Inactivation of enveloped and non-enveloped viruses on seeded human tissues by gamma irradiation. *Cell Tissue Bank.* 2012;13:401–407.
 43. Armstrong JA, Froelich EJ. Inactivation of viruses by benzalkonium chloride. *Appl Microbiol.* 1964;12:132–137.
 44. Nemo GJ, Ernest CC. Effect of visible light on canine distemper virus I. *J Bacteriol.* 1966;91:798–802.
 45. Weiss C, Clark HF. Rapid inactivation of rotaviruses by exposure to acid buffer or acidic gastric juice. *J Gen Virol.* 1985; 66(Pt 12):2725–2730.
 46. Yamamoto T, Maruyuk RG. Morphological studies of a canine adenovirus. *J. Gen. Virol.* 1968;2:191–194.
 47. Petr G, Jiran E. The effect of temperature on the hemagglutinin activity of the canine adenovirus (infectious canine hepatitis virus). *Zentralbl Veterinarmed.* 1970;17B:569–581.
 48. Yamamoto T. The effect of ion concentration and pH on the thermal stability of a canine adenovirus. *Can J Microbiol.* 1967; 13:1139–1149.
 49. Terpstra FG, Parkkinen J, Töölö H, Koenderman AHL, Ter Hart HGJ, von Bonsdorff L, Torma E, van Engelenburg, FAC. Viral safety of Nanogam, a new 15 nm-filtered liquid immunoglobulin product. *Vox Sang.* 2006;90:21–32.
 50. Roberts PL, Hart H. Comparison of the inactivation of canine and bovine parvovirus by freeze-drying and dry-heat treatment in two high purity factor VIII concentrates. *Biologicals.* 2000; 28:185–188.
 51. Valdes R, Ibarra N, Ruibal I, Beldarrain A, Noa E, Herrera N, Alemen R, Padilla S, Garcia J, Perez M, Moralez R, Chong E, Reyes B, Quinones Y, Agraz A, Herrera L. Chromatographic removal combined with heat, acid and chaotropic inactivation of four model viruses. *J. Biotechnol.* 2002;96:251–258.
 52. Klein M, Deforest A. Chemical inactivation of viruses. *Fed Proc.* 1965;24:319.
 53. Buckwold VE, Beer BE, Donis RO. Bovine viral diarrhoea virus as a surrogate model of hepatitis C virus for the evaluation of antiviral agents. *Antiviral Res.* 2003;60:1–15.
 54. van Engelenburg FAC, Terpstra FG, Schuitemaker H, Moorer WR. The virucidal spectrum of a high concentration alcohol mixture. *J Hosp Infect.* 2002;51:121–125.
 55. Morgenthaler J-J. Securing viral safety for plasma derivatives. *Transfus Med Rev.* 2001;15:224–233.
 56. Shen DT, Crawford TB, Gorham JR, McGuire TC. Inactivation of equine infectious anaemia virus by chemical disinfectants. *Am J Vet Res.* 1977;38:1217–1219.

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