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Chemical and Heat Treatment for Viral Inactivation in Porcine-Derived Gelatin

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Abstract

Background It is mandatory to demonstrate the removal or inactivation of potential viral contaminants in the manufacturing processes of pharmaceuticals derived from biomaterials. Porcine-derived gelatin is used in various medical fields, including regenerative medicine, tissue engineering, and medical devices. However, the steps of virus inactivation in the gelatin manufacturing process are poorly defined. In this study we evaluated virus inactivation in two steps of the gelatin manufacturing process.

Methods Pig skin (4.5 g), including solid pieces as intermediate products, was spiked with model viruses, including CPV (canine parvovirus), BAV (bovine adenovirus), BPIV3 (bovine parainfluenza type 3), PRV (pseudorabies virus), BReoV3 (bovine reovirus type 3), and PPV (porcine parvovirus), and underwent chemical treatment with alkaline ethanol or heat treatment at 62 °C followed by inoculation in relevant cell cultures. Viral titers in the samples were calculated based on the Behrens-Kärber method.

Results Model viruses were inactivated at different rates; however, effective inactivation of all model viruses was demonstrated by an LRV (log reduction value) over 4 by both chemical and heat treatment, and chemical treatment demonstrated rapid inactivation compared to heat treatment.

Conclusion The chemical and heat treatment steps exhibited meaningful viral inactivation capacity. They are integrated parts in the extraction and manufacturing process of porcine-derived gelatin, ensuring virus safety for use in medical applications.

Keywords Virus inactivation, Chemical treatment, Heat treatment, Gelatin

Introduction

One of the major concerns in the use of biomaterials in medical applications is the potential of viral contamination from the animal source. Epidemics of foot-and-mouth disease in cloven-hoofed animals, including cattle and pigs, have been highly contagious [1]. It has raised concerns over the use of animal-derived biomaterials including collagen and gelatin in medical applications. Gelatin is a polydisperse polymer obtained by the denaturation of collagen molecules. Briefly, the gelatin manufacturing process includes a sequence of essential steps: selecting raw materials, pre-treatment (using acids or alkalis), extraction, filtration and concentration, heat treatment, drying, milling, and packaging. Each of these steps is vital to ensure the production of a versatile and safe product, while also meeting the high-quality standards required for various applications. It is manufactured in alignment with GMP (good manufacturing practices) and accordingly with intended applications. Gelatin's intended application includes excipients (drug

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formulation), raw materials for medical devices, tissue engineering, regenerative medicines, and drug delivery [2–7]. However, the viral safety of porcine-derived gelatin is needed for medical application. Several approaches have been undertaken, including sourcing, screening of raw materials, in-process testing, and final product testing, to ensure freedom from viral contamination purposefully for patient safety [8]. Gelatin is preferably used in medical applications due to its high biocompatibility, bioabsorbability, biomimicry, biodegradability, low immunogenicity, and low material cost. To clear contaminant viruses from biologics, two processes are currently used which include virus inactivation and filtration. Virus inactivation is achieved through chemical and physical methods. The chemical method includes extreme pH and solvent treatment while the physical method includes ultraviolet and gamma irradiation and heat application. Virus retentive filtration (also called nanofiltration) is a process often used especially for small nonenveloped viruses that are not susceptible to the inactivation process [9]. Steps for virus inactivation and removal are included in the manufacturing process of pharmaceutical grade gelatin. However, there is a paucity of data on the virus safety of gelatin for medical applications. In this study we aimed to evaluate virus inactivation by chemical and heat treatment in the manufacturing process of gelatin for medical applications. The chemical inactivation process in gelatin manufacturing utilizes commonly used chemicals, such as sodium hydroxide for alkaline hydrolysis (Gelatin type B) and hydrochloric acid for acid hydrolysis (Gelatin type A), aimed at disrupting viral structures and modifying viral proteins [10]. Conversely, heat inactivation primarily involves the application of dry heat, whereby the intermediate gelatin product is exposed to elevated temperatures for a specified duration, leading to the disruption of viral proteins and denaturation due to alterations in viral structure and function [11]. Together, these treatment steps play a crucial role in ensuring the safety of gelatin.

Materials and methods

Viruses and cells

The following viruses from three different host species and cell line systems for virus titration were selected for evaluation: CPV (Canine parvovirus), BAV (Bovine adenovirus), BPIV3 (Bovine parainfluenza type 3), PRV (Pseudorabies virus), BReoV3 (Bovine reovirus type 3), and PPV (Porcine parvovirus) for chemical or heat inactivation (Table 1). Other host species were chosen as alternatives to porcine viruses because of a lack of readily available or validated assay systems. Furthermore, a selection of model viruses exhibiting varying levels of physicochemical resistance was utilized in chemical and heat treatment studies to assess the efficacy of these methods in clearing a wider array of viruses. Moreover, different model viruses were used for both chemical and heat treatment steps. Different steps in gelatin production (like chemical and heat treatment, etc.) may have varying levels of effectiveness against different viral species. Using a spectrum of viruses helps validate that all processes together provide adequate clearance of potential contaminants .

The virus–cell systems used included Crandell Rees feline kidney (CRFK), bovine testicular (BT cells), Pig-kidney-derived cells (CPK cells) all obtained from the National Veterinary Assay Laboratory, the Ministry of Agriculture, Forestry and Fisheries. Additionally, Madin-Darby canine kidney (MDCK) cells were sourced from the Kanagawa Prefectural Veterinary Diagnostic Laboratory, and African green monkey kidney-derived strain cells (Vero cells) were obtained from the National Research and Development Agency RIKEN Bio Resource Center. These systems were employed as assay systems, for CPV, BAV, PPV, BPIV, PRV and BReoV, respectively (Table 1).

Sample preparation

Pig skin (4.5 g), including solid pieces, was sampled from the initial raw material processed at the slaughterhouse and supplied by Nitta Gelatin Inc. (Osaka, Japan). This sample served as an intermediate product before undergoing chemical or heat treatment in the medical-grade gelatin manufacturing process. The sampled product had a gel-like appearance with solid pieces. Upon receipt, it was placed in a container and stored in the refrigerator until it could be mixed with a model virus.

Table 1 Characteristics of relevant virus models

Treatment	Virus	Host species	Genome type	Envelope	Size	Physicochemical resistance	Cell lines
Chemical	Parvovirus (CPV)	Canine	DNA	Nonenveloped	18–24 nm	High	CRFK
	Adenovirus (BAV)	Bovine	DNA	Nonenveloped	70–90 nm	Medium	BT
	Parainfluenza virus (BPIV)	Bovine	RNA	Enveloped	100–200 nm	Low	MDCK
Heat	Parvovirus (PPV)	Porcine	DNA	Nonenveloped	18–24 nm	High	CPK
	Reovirus (BReoV)	Bovine	RNA	Nonenveloped	60–80 nm	Medium	Vero
	Pseudorabies virus (PRV)	Porcine	DNA	Enveloped	120–200 nm	Low	Vero

Cytotoxicity tests

To verify whether there was any impact of the test substance on the cells, cytotoxicity tests were performed.

Briefly, 0.11 mL of 2% FBS-added Eagle's MEM medium (Eagle's MEM medium, 2% FBS, 0.3% tryptose phosphate broth, 0.1% sodium hydrogen carbonate aqueous solution) was added to 1 g of the test sample, and with scissors, the sample was finely chopped and transferred to a new tube followed by an addition of 10 mL of 2% FBS-added Eagle's MEM medium. After mixing with a mixer, a stir bar was inserted, and a stirrer was used to homogenize it. After a low-speed centrifugation (1000 x g, 10 min, 4 °C) using a centrifuge (Multibridge cooling centrifuge 8900: Kubota Corporation Co. Ltd.) a 0.45 µm filter was used to filter the supernatant. The obtained sample was serially diluted tenfold with 2% FBS-added Eagle's MEM medium and inoculated with various test cells seeded in a 24-well plate. During the cultivation period, plates were observed to check any abnormalities in the cells compared to the positive control, and judgments were made accordingly [12].

Virus culture

The following viruses were used in combination with the cell systems described above for growth and harvest, at the specified titers: CPV (10^7.5 TCID50/mL) supplied by the University of Tokyo; BAV (10^8.0 TCID50/mL) and BReoV3 (10^8.5 TCID50/mL) supplied by the Japan Veterinary Products Association; and BPIV3 (10^9.0 TCID50/mL), PRV (10^8.6 TCID50/mL) and PPV (10^7.9 TCID50/mL) supplied by the Kanagawa Prefectural Veterinary Diagnostic Laboratory, as previously described [13–18].

CPV and PPV:

Briefly, cells were suspended in 5% FBS-EMEM at a concentration of 3×10^5 cells/mL, and 15 mL of this suspension was added to a 75 cm^2 flask. To this, 1 mL of each virus solution (10^3 TCID50/mL) was added, and the flask was incubated in a 37 °C, 5% CO2 incubator for 1 day. After the incubation, the culture supernatant was removed, and 15 mL of 2% FBS-EMEM was added, followed by an additional 6 days of incubation. After this period, the flasks were frozen at -80 °C, thawed, and the cells and culture supernatant were collected and centrifuged at 3000 rpm for 10 min at 4 °C. Only the supernatant was collected, aliquoted, and frozen at -80 °C as a virus solution.

BAV, PIV, BReoV and PRV:

Cells were cultured subconfluently in 75 cm^2 flasks. After removing the cell culture supernatant, the cells were washed once with PBS. Then, 1 mL of a 10^6 TCID50/mL virus solution was added to each flask and allowed to adsorb for 1 h at 37 °C in a 5% CO2 incubator. The virus solution was removed, and 15 mL of 2% FBS-EMEM was added, followed by incubation at 37 °C in a 5% CO2 incubator for the following durations: PRV for 2 days, BPIV for 4 days, and BAV and BReoV3 for 7 days. After incubation, the flasks were frozen at -80 °C, thawed, and the cells and culture supernatant were collected and centrifuged at 3000 rpm for 10 min at 4 °C. Only the supernatant was collected, aliquoted, and frozen at -80 °C as a virus solution.

Virus spiking

In brief, for virus inactivation by chemical treatment of CPV, BAV, and BPIV, 4.5 g of the test sample was spiked with 0.5 mL of working seed virus followed by the addition of 11.1 mL of alkaline-ethanol solution, and the resulting mixture was stirred at a refrigeration temperature for a treatment time including 1, 3, 6, and 24 h. Following the treatment, the supernatants were isolated and 11.1 mL of the prewash culture medium composed of 2% FBS-added Eagle's MEM medium was immediately added to the culture and after a brief agitation, the whole medium was collected to recover the virus.

For virus inactivation by heat treatment of PPV, BReoV, and PRV, briefly, 4.5 g of the test sample was inserted into respective tubes and spiked with 0.5 mL working seed virus followed by an insertion of a temperature logger sensor to perform temperature monitoring. The tubes were then placed in a thermoregulator at 62 °C, and heat treatment was performed for 1, 3, or 5 h. Then, the various samples were removed and immediately stored in subzero temperature water.

To recover PRV and BReoV, 45 ml of 2% FBS-added Eagle's MEM medium was added to the treated samples. For PPV, 5% FBS-added Eagle's MEM medium (Eagle's MEM medium, 5% FBS, 0.3% tryptose phosphate broth, 0.1% sodium hydrogen carbonate aqueous solution) was added to the treated samples. After mixing well with a mixer, a stir bar was inserted and a stirrer was used to homogenize it followed by a low-speed centrifugation (1000 x g, 10 min, 4 °C) and a 0.45 µm filter was used to filter the supernatant.

The treatment times were chosen based on common pretreatment periods for chemical and heat steps in the gelatin manufacturing process. Chemical treatments may last up to 72 h, while heat treatments may last up to 48 h. These durations can be set or adjusted to control the final properties and functionalities of the desired product [19].

Recovery of the spike virus

Chemical treatment

The 0 h recovery of spiked virus was conducted as follows. A 0.5-g (0.5 mL) working seed solution of a spike virus was added to 4.5 g of the test substance. Subsequently, 11.1 mL of the culture medium (2% FBS-added Eagle's MEM medium), was added to the mixture and

no treatment with alkaline-ethanol solution was applied. The added medium was recovered immediately, and another 11.1 mL of the same medium was added. After a brief agitation, the entire amount of the medium was collected and combined with the solution collected earlier.

Heat treatment

The 4.5 g of the test substance was spiked with 0.5 mL of sample for each virus. Following this, it was immediately chopped finely with scissors and transferred to a tube, after which 45 mL of 2% FBS-added eagle MEM medium or 5% FBS-added eagle MEM medium was added. After mixing this with a mixer, a stir wrapper was inserted, and a stirrer was used to homogenize it. Low-speed centrifugation (1,000×g、10 min, 4 °C) was then performed using a centrifuge, and the liquid obtained after filtering this supernatant with a 0.45 μm filter.

Evaluation of validity

The sample stock solution for each of the virus type was 10-fold diluted, and the virus quantity in the samples were measured using the methods below. The mean values from the values repeatedly measured 3 times for each measurement date were sought in order to obtain measurement values for all of the measurement data.

A check was performed on whether the values repeatedly measured 3 times for each test were within the range of the respective (mean value of the values repeatedly measured 3 times for each measurement date±1) log. Additionally, in case the mean value of the values repeatedly measured 3 times for each measurement date were within the range of the (working seed virus quantity±1) log, it was assessed that the spike virus was sufficiently recovered, and that the test method was valid.

Sample inoculation

Cells were suspended at a concentration of approximately 5×10^5 cells/mL in the culture medium (5% FBS-added Eagle's MEM medium) and seeded at 0.5 mL/well. All samples were subjected to tenfold serial dilution with culture medium (2% FBS-added Eagle's MEM). The various spiked solutions were inoculated at 0.1 mL/well in 5 wells of the 24-well plate and left to stand for 1 h (BAV, BPIV, PRV, BReoV, and PPV) or overnight (CPV) in a 5% carbon dioxide incubator at 37 °C. The culture fluids were removed by aspiration, 0.5 mL of fresh culture medium was added, and the cells were incubated at 37 °C in a 5% CO2 atmosphere for 7 days.

Virus detection

i. CPV detection

A volume of 0.025 mL of the culture supernatant was collected from each well and transferred to a 96-well V-bottom plate. To this, the same volume of borate-buffered saline containing bovine serum albumin (0.7% sodium chloride, 0.3% boric acid, 0.1% sodium hydroxide) and 0.05 mL of 0.5% porcine red blood cell suspension (0.5% porcine red blood cell suspension) was added successively, and the mixture was incubated overnight at 4 °C. Then, HA was determined (by observing the presence or absence of teardrop-shaped streaming of the red blood cells). Cultures showing HA were considered virus-positive, and the virus content in the samples was calculated based on the Behrens-Kärber method [20].

ii. BAV, PRV, BReoV detection

Cells were observed during the 7 days of incubation, and the virus contents in the samples were calculated based on the Behrens-Karber method using the presence or absence of CPE on the 7th day of culture as an indicator.

iii. PPV detection

On the final day of cultivation (7th day), 0.05 mL of the cultivated supernatant was sampled from each well and migrated to a 96-well, U-shaped plate. To this, 0.05 mL of 0.5% chicken red blood cell suspension adjusted using a veronal buffered saline solution (Lonza Ltd.) was added and left to stand at room temperature for 60 min to observe whether hemagglutination (HA response) was present. Wells in which the HA response was observed were considered virus-positive, and the virus quantity in the sample was calculated based on the Behrens-Kärber method.

Positive controls

For the positive control (working seed), the virus quantity was repeatedly measured thrice for each measurement date and each test. The measurement of the virus quantity was confirmed to be within the (working seed virus quantity±1) log range before treatment. Spiked samples were separately prepared as run controls and left to stand at room temperature until the final processing time.

Virus reduction

The virus reduction capacity in the treatment steps was determined based on the LRV (log reduction value) calculated from the mean of triplicate measures at each point.

LRV: log (mean virus content from triplicate measurements at 0 h - mean virus content from triplicate measurements at end of processing time).

Data analysis

The virus contents in the sample were calculated based on the Behrens-Kärber method using the presence or absence of CPE or haemagglutination on the 7th of the culture as an indicator. Data analysis was conducted using Prism software 8.0 (GraphPad, San Diego, CA, USA), and a P value <0.05 was considered statistically significant. Each experiment was carried out in triplicate, repeated once, and the graphs show the means and standard deviations of LRV.

Results

For the chemical treatment, cytotoxicity was observed in cells inoculated with untreated virus samples, and a detection limit of 1.5 $\log_{10}$ $TCID_{50}$/mL was determined compared to heat treatment where it was not observed and a detection limit of 0.5 $\log_{10}$ $TCID_{50}$/mL was determined (data not shown).

After confirming the recovery efficiency of each virus model (Table 2), virus inoculation was performed and the quantity of virus recovered was determined to confirm the stability of the virus during the experiment. Following deliberate virus spike with relevant model viruses, some viruses were inactivated at different rates; however, effective inactivation of all model viruses with an LRV over 4 were observed in both chemical or heat treatment, and viruses subjected to chemical treatment demonstrated rapid inactivation compared to those under heat treatment. The model seed working viruses remained stable throughout the evaluation test for chemical treatment. The inactivation of BPIV, BAC, and CPV in the chemical treatment exhibited the same kinetic which was a rapid decreased of infectivity of these spiked viruses after an hour of treatment to undetected level. On another hand, in the 5-hour heat treatment, PRV decreased significantly over time, approaching an LRV of 1.5, while BReoV exhibited a strong and consistent downward trend in LRV and PPV decreased sharply after an hour of treatment and the rate of decrease appears to slow down after one hour to 5 h-Chemical treatment. (Figs. 1A-C), and heat treatment (Fig. 2A-C).

Table 2 Validation test of virus stabilities following the virus spike of sample material

Virus	Spiked (log10$TCID_{50}$/mL)	Recovered (log10$TCID_{50}$/mL)	Recovery error
Canine parvovirus (CPV)	7.5 ± 0.1	5.6 ± 0.2	1.9
Bovine adenovirus (BAV)	7.9 ± 0.0	6.2 ± 0.1	1.7
Bovine parainfluenza (BPIV)	9.0 ± 0.1	7.0 ± 0.2	2.0
Pseudorabies virus (PRV)	8.6 ± 0.0	7.8 ± 0.0	0.8
Bovine reovirus (BReoV)	8.5 ± 0.14	8.7 ± 0.07	0.2
Porcine parvovirus (PPV)	7.9 ± 0.0	7.9 ± 0.07	0.0

The spike virus was added at a 1:10 ratio. For the validation samples, the virus quantity was measured after processing the emulsion, which included a final dilution factor of 10. The validation samples, after correction, accounted for a 100-fold increase by adding '2' to the value to adjust for the dilution

The Recovery Error refers to the absolute value of the difference between the virus quantity measured in the supernatant and the amount of spiked virus added to the samples, as calculated based on the spike virus (working sheet)

Discussion

In the gelatin manufacturing process, chemical treatment either with alkaline or acid and heat treatment are integrated parts of the extraction process. The application of chemical treatment (alkaline) or heat treatment on in-process gelatin samples resulted in titer reductions of all virus models, with the LRV reaching 4 within 1 h of any treatment. The reduction factor is a relevant indicator in describing the viral reduction potential. An LRV of 4 or more is indicative of a clear effect for a particular virus under investigation [21, 22]. PPV represents a worst-case model for assessing virus removal by filtration due to its small size and by heat inactivation due to lack of a viral envelope, requiring higher temperature or time for degradation [9, 23]. In this study, PPV exhibited a higher resistance to heat treatment compared to other model viruses, corroborating results of previous heat inactivation studies where it required higher temperature or a longer time to reach an LRV of 4 [24]. Nevertheless, like PRV and BReoV, PPV was also inactivated by heat treatment, demonstrating a log reduction value (LRV) of more than 4 $\log_{10}$. Heat inactivation is mechanistically achieved by denaturing the secondary structures of proteins and other molecules such as nucleic acids (including viral RNA or DNA), resulting in impaired molecular functions, especially host-cell binding, or rendering structural proteins susceptible to protease attack [11, 25]. Heat treatment appears to be associated with slower virus inactivation rate compared to Chemical treatment [26]; this effect is also demonstrated in our studies. The chemical treatment exhibited rapid inactivation of all model viruses within 1 h of treatment, resulting in LRVs above 4 regardless of the model virus, including Parvovirus. Previous studies have also reported that for porcine-derived collagen-based material, a rapid viral inactivation with peracetic acid/ethanol for enveloped and nonenveloped viruses including Parvovirus [27], . In this study, the overall chemical treatment time was 24 h to mimic a typical chemical treatment step in the manufacturing process of gelatin. This most likely infers that virus inactivation is achieved as the chemical treatment time continues

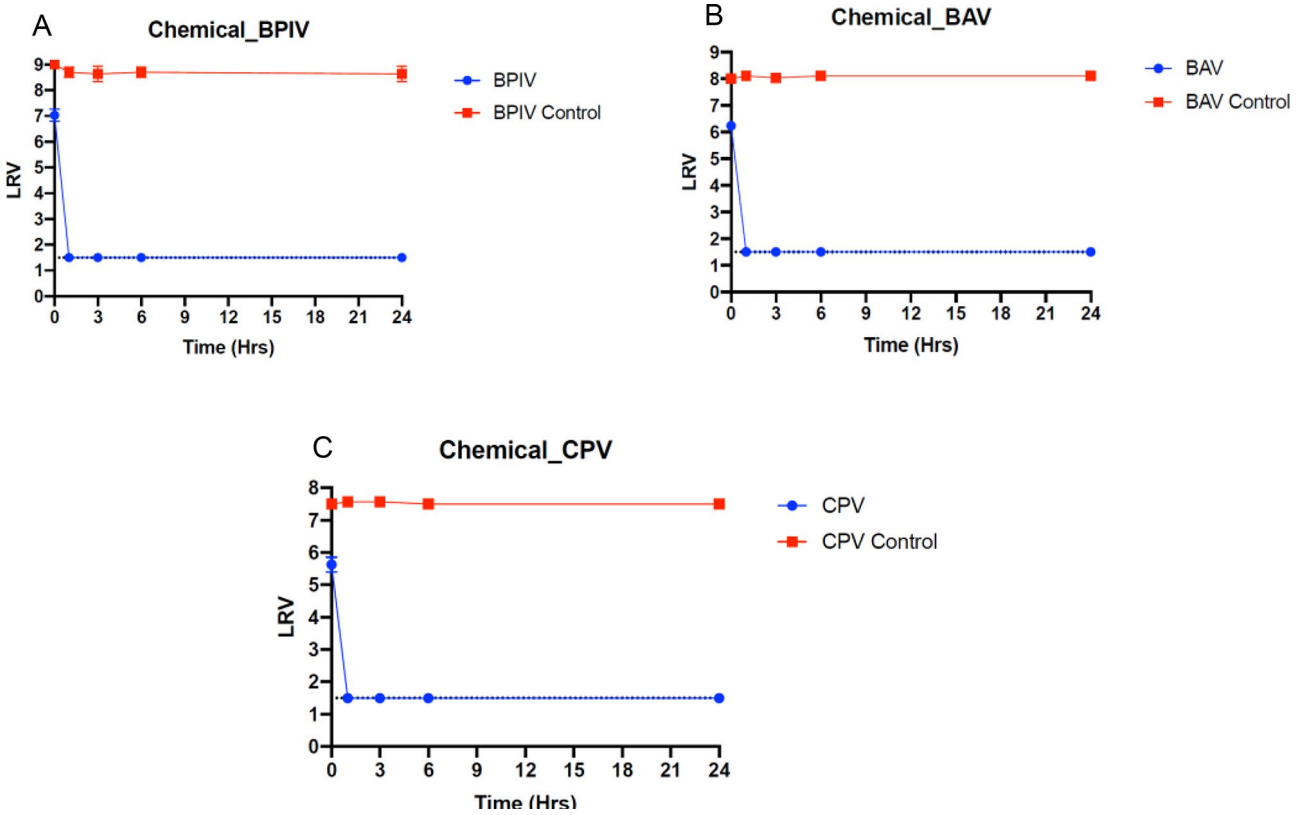

Fig. 1 (**A**) Kinetics of BPIV chemical inactivation. (**B**) Kinetics of BAV chemical inactivation. (**C**) Kinetics of CPV chemical inactivation

beyond hours. Chemical inactivation causes damage to virus functionality, including protein and genome degradation, resulting in loss of proteins and genome-mediated functions [10, 28].

The heat and chemical inactivation processes incorporated in the gelatin manufacturing process constitute at least two orthogonal steps used to achieve a complementary approach for virus clearance [22, 29]. Moreover, a virus safety process for a licensed product is validated through a model of virus clearance study conducted with at least 4 viruses including small-size nonenveloped viruses such as PPV, CPV, or MVM (minute virus of mice), and an LRV of 4 or higher; models meeting these requirements are perceived as a robust and effective safety measure [30].

The chemical and heat inactivation steps of the virus safety process do not alter gelatin properties of the final product. On the other hand, these two steps alone in the gelatin manufacturing process may not necessarily result in their effectiveness in virus inactivation. The virus reduction effectiveness requires the consideration of various parameters, many of which were covered in this study including the LRV achieved, the time-dependence of inactivation, and the limits of assay sensitivities. Moreover, the gelatin process includes filtration steps with filter sizes as small as 0.22 µm to achieve an exceptional level of sterility in solutions. In this study we evaluated the inactivation steps in a pigskin-derived gelatin manufacturing process model. However, other material sources including fish and cow are used for gelatin production with different manufacturing processes and each final product may require a different study design for the evaluation of virus safety. Meanwhile, potential threat for gelatin from sourcing such as fish and cow are primarily bacteria or prions respectively, and virus clearance is most likely not of concern, especially for medical devices for In Vitro Diagnostic Devices. However, current unknown risks of viral disease transmission from fish such as cod fish can be uncover through new technologies including Next Generation Sequency, Metagenomics and CRISPR-based diagnostics. The limitations of this study included the use of lessrelevant viruses for the process of evaluation due to the unavailability of more-relevant viruses. However, appropriate specific model viruses [22] were used as substitutes as they are closely related to the known viruses based on genus or family lineage. Additionally, the potential of model virus reaction with the sample was not evaluated, and the reduction factors were expressed on a logarithmic scale, indicating that the virus infectivity of the residues will not be eliminated completely. Virus clearance evaluation purposefully provides a level of assurance that undetected viruses or those

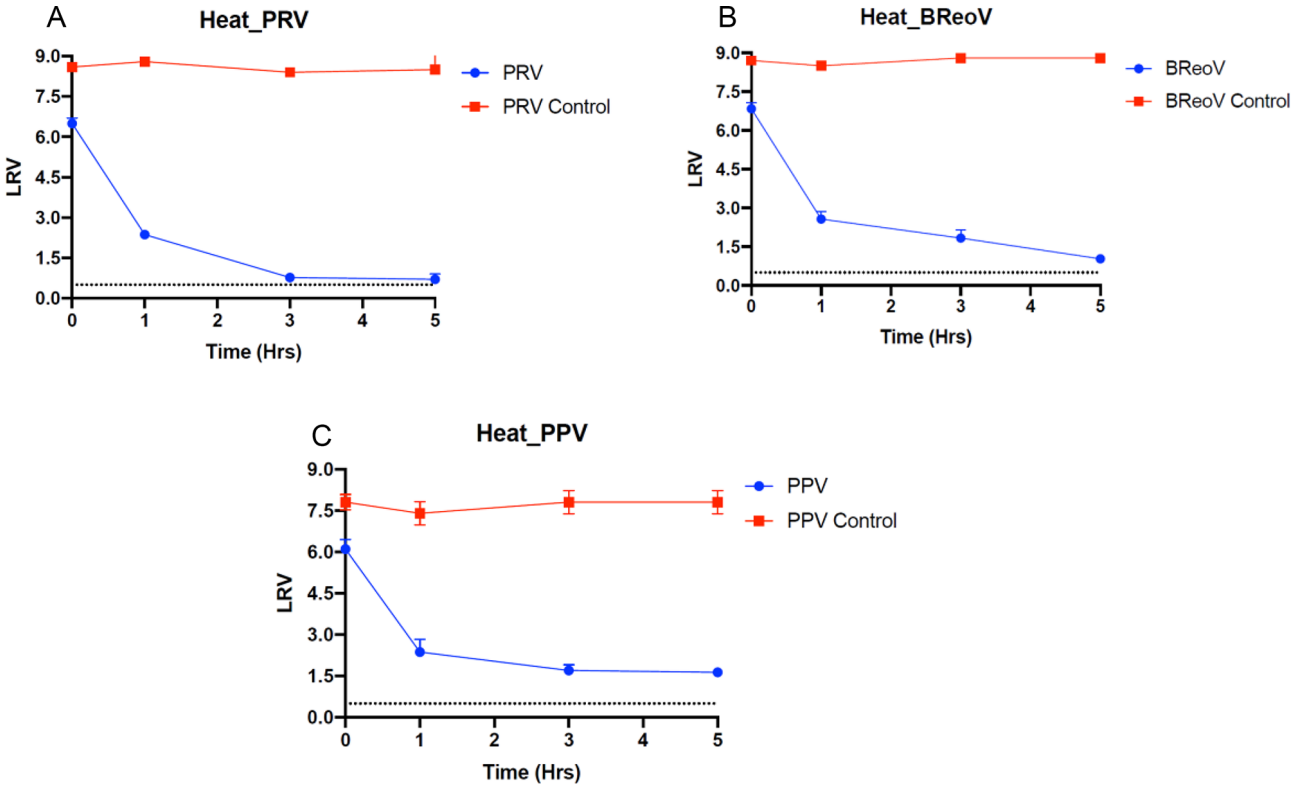

Fig. 2 (**A**) Kinetics of PRV heat inactivation. (**B**) Kinetics of BReoV heat inactivation. (**C**) Kinetics of PPV heat inactivation. Each data point shows the mean from triplicate measurements. The positive control (working seed) was measured three times for each measurement date and each test

contaminating the production process will be cleared, and the gelatin manufacturing process clears viruses through some process steps including the inactivation steps.

Conclusion

Regardless of differences of resistance in model viruses including small size nonenveloped viruses, virus reduction capacity is achieved in the gelatin manufacturing process by both chemical and heat treatment steps.

Abbreviations

CPV	Canine parvovirus
BAV	Bovine adenovirus
BPIV3	Bovine parainfluenza type 3
PRV	Pseudorabies virus
BReoV3	Bovine reovirus type 3
PPV	Porcine parvovirus
LRV	Log reduction value
CRFK	Crandell Rees feline kidney
BT	Bovine testicular
MDCK	Madin-Darby canine kidney
CPK	Pig-kidney-derived strain cells
FBS	Fetal Bovine Serum
MEM	Minimum Essential Medium
HA	haemagglutination assay
CPE	Cytopathic effect
$TCID_{50}$	Median Tissue Culture Infectious Dose

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Author contributions
FK study protocol development and manuscript drafting and review. MS Sample preparation and data management. KK sample preparation and data management. MK data management and project administration YH conceptualization, study protocol development, and study supervision. All authors read and approved the final manuscript.

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Data availability
The data and materials used in this study are available upon request from the corresponding author. All relevant data supporting the findings of this research are included within the manuscript. Any additional materials or datasets can be provided to interested parties following a reasonable inquiry.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
All authors are employees of Nitta Gelatin Inc.

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