

# The effect of extended storage on virus survival in feed

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## Summary

Extended feed storage to reduce the risk of virus survival has not been tested experimentally. Five ingredients inoculated with porcine epidemic diarrhea virus, porcine reproductive and respiratory syndrome virus-174, and Senecavirus A were stored indoors at 20°C or outdoors in Minnesota winter conditions. After 30 days, outdoor samples contained infectious virus, while indoor samples did not.

**Keywords:** swine, feed ingredients, extended storage, viral diseases, demonstration project

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## Resumen - El efecto del almacenamiento prolongado sobre la supervivencia de virus en el alimento

No se ha probado experimentalmente el almacenamiento prolongado de alimentos para reducir el riesgo de supervivencia de virus. Cinco ingredientes inoculados con el virus de la diarrea epidémica porcina, el virus 174 del síndrome reproductivo y respiratorio porcino, y el Senecavirus A se almacenaron al interior a 20°C o al aire libre en condiciones invernales de Minnesota. Después de 30 días, las muestras al aire libre contenían virus infecciosos, mientras que las muestras almacenadas en el interior no.

## Résumé - Effet de l'entreposage prolongé sur la survie de virus dans les aliments pour animaux

Un entreposage prolongé des aliments pour réduire le risque de survie de virus n'a pas été testé expérimentalement. Cinq ingrédients inoculés avec le virus de la diarrhée épidémique porcine, le virus du syndrome reproducteur et respiratoire porcine-174, et le Senecavirus A ont été entreposés à l'intérieur à 20°C ou à l'extérieur dans les conditions hivernales du Minnesota. Après 30 jours, les échantillons extérieurs contenaient des virus infectieux, contrairement aux échantillons intérieurs.

In 2014, feed and feed ingredients were proposed as vehicles for the transport and transmission of porcine epidemic diarrhea virus (PEDV) from China to the United States.<sup>1-3</sup> This hypothesis has since been expanded across multiple viruses, such as Senecavirus A (SVA), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus, pseudorabies virus, and African swine fever virus.<sup>4-6</sup> These studies have also repeatedly confirmed that certain feed ingredients, such as soy-based products, appear to promote virus survival over time.<sup>1-6</sup> To mitigate this risk, the North American swine industry has attempted to reduce virus viability in feed using a variety of approaches, including mechanical

reduction (flushing and sequencing),<sup>7,8</sup> heat treatment,<sup>9</sup> pelleting,<sup>10</sup> chemical mitigation,<sup>11,12</sup> and extended storage. This latter approach is a critical component of the policy of Responsible Imports, a science-based protocol to safely introduce essential feed ingredients from high-risk countries using extended periods of storage under climate-controlled conditions to reduce virus viability.<sup>13</sup> Along these lines, import requirements of select feed ingredients from countries endemically infected with African swine fever virus has been adapted by the Canadian Food Inspection Agency<sup>14</sup> and wide-scale voluntary application of Responsible Imports has occurred across the US swine industry.<sup>15</sup> These protocols involve the storage of

imported feed ingredients into designated facilities for a predetermined period and held under a controlled temperature, prior to movement to mills and farms. However, while widely applied, these protocols have been primarily based on mathematical estimates of half-life, not experimentally derived data.<sup>16</sup>

To address this limitation, we designed an experiment using an approach taken from the social sciences known as the “demonstration project.” A demonstration project is defined as a means of promoting innovations and disseminating best practice through the development and analysis of a live project, undertaken in natural settings that resemble non-experimental, real-world conditions.<sup>17</sup>

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This approach has been used to help build an evidence base to support industry improvements, as historically, lessons learned from demonstrations, through the rigors of scientific research, have resulted in large-scale adoption and major shifts in aims, styles, and resources. Therefore, the purpose of this study was to design a demonstration project to test the effect of an extended storage protocol on the survival of swine viral pathogens in feed ingredients under real-world conditions. The study was based on the hypothesis that controlling temperature during storage would enhance the success of the protocol.

## Materials and methods

### Ethical statement

Animals in this study were managed in accordance with the institutional animal care and use guidelines observed by the investigators' ethical review board, Pipestone Applied Research IACUC, trial number 2020-02.

### Sample preparation

Viruses selected for this study included PRRSV-174, PEDV, and SVA while ingredients included conventional soybean meal, organic soybean meal, choline chloride (60%, no corn cob carrier), lysine HCL (78.8% minimum lysine, no carrier) and vitamin A (1,000,000 IU with porcine coated gelatin).<sup>1-6</sup> As previously defined, conventional soybean meal contained a low fat (1%-2%) and high protein (46%-47%) content, while the organic product had higher fat (6%-7%) and lower protein content (44%-45%).<sup>3,4</sup> Samples of each ingredient were obtained from local mills and were not irradiated prior to initiating the study. Four, 30-g allotments of the 5 ingredients were weighed into individual 50 mL mini-bioreactor tubes with vented caps (Corning Inc) for a total of 20 samples, providing 4 replicates per each of the 5 ingredients. This was defined as a sample set. For preparation of the viral inoculum to be used to spike ingredients, a single batch of viral inoculum, containing a mixture of all three viruses was prepared. Specifically, each virus was diluted in 100 mL minimum essential medium (MEM, Sigma-Aldrich) to a concentration of  $1 \times 10^5$  50% tissue culture infectious dose per mL per virus. All 3 viruses were then mixed (three viruses for a total of 300 mL) followed by an addition of 200 mL MEM, to bring the total volume to 500 mL. This concentration was based on a previous

publication documenting this level of PEDV in feed bin samples from index farms in 2014.<sup>1</sup> Each of the 20 samples were then individually spiked with a 2 mL aliquot from the viral mixture to measure viral load at the end of the 30-day study period. Inoculums were injected directly into the center of each 30-g ingredient sample using a 3 mL syringe with an 18-gauge, 3.81 cm needle. In addition to the 20 spiked samples, 2 positive controls (stock virus mixture in the tube in the absence of feed), 2 negative controls (30g of conventional soybean meal, no virus), and 1 contamination control (empty tube, no feed, no virus) were included in the design. The purpose of the positive controls was to determine whether viruses could survive in the absence of a feed matrix, while the negative controls and the contamination control were included to validate whether cross-contamination occurred or not. Duplicate sample sets, each consisting of 20 samples (4 tubes of each of the 5 ingredients) plus controls resulting in a total of 25 tubes per sample set, were included in this study. The purpose of the duplicated samples was to assess the repeatability of the results.

### Storage conditions

Based on feedback from the US industry (S. Dee, DVM, personal communication, 2018-2019), a protocol involving a 30-day storage period at a temperature of 20°C was selected for this study. The study was conducted in the basement of the principle investigator's home in west-central Minnesota, beginning January 31, 2020 and ending February 29, 2020. For outdoor storage, one sample set (as defined) was placed 2 m outside the home's basement entrance, allowing for exposure to natural conditions. For indoor storage, the second sample set was placed in a designated room inside the home, allowing for exposure to climate-controlled conditions generated by the household heating system. During the indoor evaluation, the thermostat was set at 20°C and was programmed to remain constant 24 hours each day of the 30-day storage period. To record environmental conditions during the 30-day period, a data logger (RC-51H, ELITech) was placed alongside both sample sets and temperature and relative humidity (RH) was recorded every 15 minutes each day.

### Diagnostic testing

Following completion of the 30-day storage period, samples were evaluated for the presence of viral RNA by polymerase

chain reaction (PCR) and for viability by swine bioassay. For PCR, samples were tested at the South Dakota State University Animal Disease Research and Diagnostic Laboratory (SDSU ADRDL) using published methods.<sup>1-3</sup> For bioassay, pigs were housed in the Pipestone Applied Research biosafety level 2 facility. The bioassay involved 50 three-week old pigs, which were housed in three rooms and originated from a farm known to be naïve for PRRSV, PEDV, and SVA. The first room was designated for outdoor storage assessment and the second for indoor storage assessment. In each room, pigs were penned according to ingredient (5 pens, 4 pigs/pen, 20 pigs/room). Control pigs were housed in the third room. Five pigs were used as outdoor storage controls and placed in the third room which contained 6 pens. The 2 positive-control pigs were placed in the first pen and the 3 negative-control pigs in the second pen. Five additional pigs were designated as indoor storage controls and placed in the third room with 2 positive-control pigs in the fifth pen and 3 negative-control pigs in the sixth pen. The purpose of the positive-control pigs was to determine whether viable virus was present in the positive-control samples (virus, no feed matrix) from outdoor samples and indoor samples. The purpose of the negative-control pigs was to determine whether cross-contamination of the viruses had occurred during sample handling or during storage from outdoor samples and indoor samples. Pen dividers and empty pen spaces between animal groups were used to eliminate nose-to-nose contact and minimize the chances of indirect transmission between pens. For preparation of the bioassay inoculum, each 30-g sample from the 5 feed ingredients in each sample set at 30 days post inoculation was transferred to separate 250 mL conical tubes, followed by the addition of 60 mL of sterile saline. Each sample was then homogenized and centrifuged at 4000g for 10 minutes, with supernatant decanted into a clean 50 mL tube and recentrifuged at 4000g for 10 minutes. Supernatant was then decanted into 10 mL tubes and frozen at -80°C, in preparation for inoculation. All pigs were inoculated with a 2 mL sample via the intramuscular route for assessment of PRRSV and SVA infectivity and 2 mL via the oral route for assessment of PEDV infectivity. A mixed virus sample was used for ease of handling based on previous experience.<sup>18</sup> Rectal swabs and blood samples were

then collected at days 0, 7, and 14 post inoculation and submitted to the SDSU ADRDL for analysis.

## Results

### Sample integrity during storage

During the 30-day storage period, outdoor samples quickly froze post placement and remained frozen until processed. In contrast, indoor samples did not freeze, although there appeared to be some loss of volume in the positive-control samples (stock virus in MEM, no feed), possibly due to evaporation and drying, secondary to the warm, dry conditions of the storage area.

### Presence of viral nucleic acid in feed

Mean viral load at day 30 post feed inoculation across the 3 viruses and the 5 ingredients, inclusive of controls, are summarized in Figure 1A (outdoor storage) and 1B (indoor storage). In both storage methods, PRRSV, PEDV, and SVA RNA were detected across all 5 ingredients, with some degradation of viral nucleic acid observed. In addition, viral RNA was detected in positive-control samples, but not in negative controls.

### Viability assessment

Prior to inoculation, all pigs were confirmed to be naïve to all three viruses via serum samples and rectal swabs collected on day 0. Following inoculation of pigs with 30-day outdoor storage samples, PRRSV and SVA RNA was detected by PCR in serum samples and PEDV RNA in rectal swabs collected at day 7 and day 14 post inoculation from bioassay pigs in the organic soybean meal group (4 of 4 pigs), the conventional soybean meal group (4 of 4 pigs), the vitamin A group (4 of 4 pigs), the lysine group (4 of 4 pigs), and the choline group (4 of 4 pigs). In addition, clinical signs suggestive of PRRSV (dyspnea, hyperthermia), PEDV (diarrhea), and SVA (lameness) were observed across all groups. Positive controls (2 of 2 pigs) were bioassay positive, while negative controls (3 of 3 pigs) were bioassay negative. In contrast, following inoculation of pigs with 30-day indoor storage samples, no evidence of PRRSV, PEDV, or SVA RNA was detected by PCR in serum and rectal swab samples from any of the 20 bioassay pigs. In addition, clinical signs suggestive of PRRSV, PEDV, and SVA were not observed in any

groups. Positive controls (2 of 2 pigs) were bioassay negative, as were all 3 negative controls.

### Temperature and RH data

Over the course of the 30-day period, the mean outdoor temperature was  $-8.8^{\circ}\text{C}$  with a maximum of  $-4^{\circ}\text{C}$  and a minimum of  $-14.7^{\circ}\text{C}$ . The mean outdoor RH was 77%, with a maximum of 88% and a minimum of 62%. Over the same period, the mean indoor temperature was  $20.1^{\circ}\text{C}$ , with a maximum of  $20.4^{\circ}\text{C}$  and a minimum of  $19.8^{\circ}\text{C}$ , while the mean RH was 35%, with a maximum of 37% and a minimum of 34%.

## Discussion

The purpose of this study was to conduct a demonstration project to evaluate whether extended storage in a climate-controlled environment would reduce the risk of virus-contaminated feed versus storage outside during a Minnesota winter. Under the conditions of the study, these data demonstrate that across the 5 feed ingredients evaluated, the indoor storage protocol successfully inactivated 3 significant pathogens of swine, including PRRSV-174, PEDV, and SVA. In contrast, all 3 viruses survived in all 5 ingredients following external storage. Based on the environmental data collected during indoor storage, the storage area remained at a consistently warm temperature (mean =  $20.1^{\circ}\text{C}$ ) with a low RH (mean = 35%) throughout the 30 days. In contrast, the outdoor environment was generally cold (mean =  $-8.8^{\circ}\text{C}$ ) and moist (RH = 77%) and varied over time. These contrasting environmental parameters most likely played a significant role in the ability of the 3 viruses to survive during their respective storage periods.

While the results are promising, this study had its share of acknowledged strengths and limitations. Strengths included the novelty of the demonstration project (real-world storage conditions), the use of multiple replicates per feed ingredient, and the inclusion of negative controls to confirm that cross-contamination did not occur. A significant limitation of the study was that it was conducted only once, and no evaluation of repeatability or consistency of the outcomes can be predicted. This is important as data from a single replication does not allow us to determine the protocol efficacy in all cases, ie, we cannot say that the protocol tested will eliminate

virus infectivity 100% of the time. Other limitations include the use of a single viral concentration to inoculate the ingredient samples and the use of a small sample size, and small quantities (30g) of 5 feed ingredients spiked with relatively large volumes of liquid inoculum. While small quantities were used to minimize the risk of false negative results, studies are underway to repeat this project using larger volumes of ingredients inoculated with proportionately representative volumes of liquid inoculum. Finally, this study evaluated an indoor storage protocol that only incorporated one time and one temperature setting, and the study was conducted at one location in the US during one season of the year. Further studies should be conducted utilizing different conditions to develop a database comparing success of varying extended storage protocols across different environments, as well as repeatability of the results.

Under the conditions of this study, the results demonstrated that an extended storage period of 30 days at a temperature of  $20^{\circ}\text{C}$  was effective at reducing the viability of 3 significant viral pathogens of pigs across multiple feed ingredients. It is hoped that this information will support further application of extended storage procedures on farms and in mills. Finally, further studies should be conducted using other significant foreign animal disease pathogens such as African swine fever virus and foot-and-mouth disease virus to further justify the additional costs and logistics of implementing this approach.

## Implications

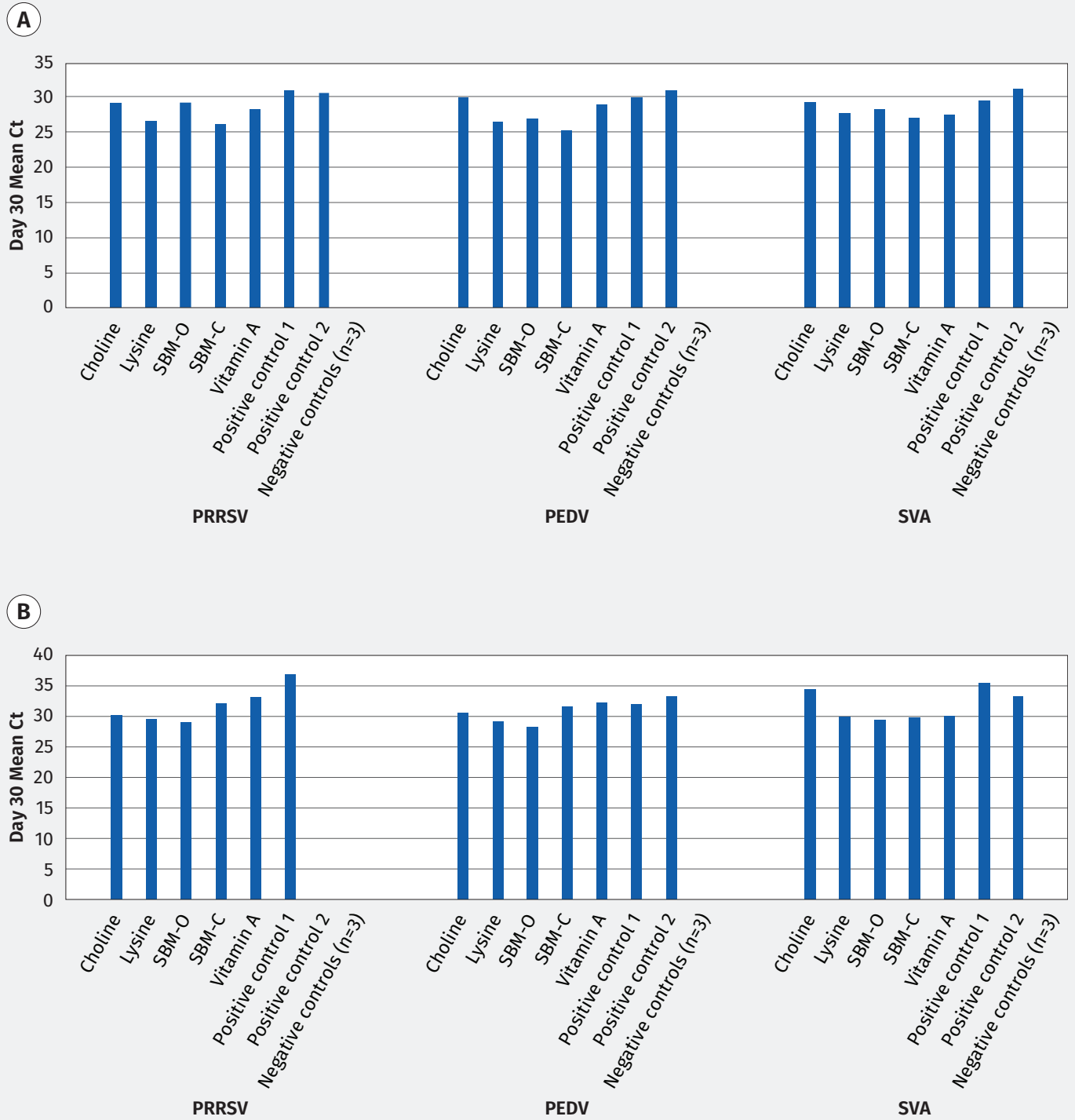
Under the conditions of this study:

- A specific protocol of extended storage inactivated PEDV, PRRSV, and SVA.
- All viruses survived in all 5 ingredients stored in cold weather conditions.
- Extended feed storage should involve a climate-controlled environment.

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**Figure 1:** Summary of mean Ct values by virus and ingredient on day 30 post inoculation under A) outdoor storage or B) indoor storage, along with positive and negative controls. PCR-negative samples were given a value of “0”. Ct = cycle threshold; PCR = polymerase chain reaction; SBM-O = organic soybean meal; SBM-C = conventional soybean meal; PRRSV = porcine reproductive and respiratory syndrome virus; PEDV = porcine epidemic diarrhea virus; SVA = Senecavirus A.





## Conflict of Interest

None reported.

## Disclaimer

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