SWINE HEALTH INFORMATION CENTER
FINAL RESEARCH GRANT REPORT FORMAT

Project Title and Project identification number: Improving the efficacy of extended storage for reducing the risk of viral-contaminated feed through a better understanding of the effective ambient temperature.

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Industry Summary: Viruses of veterinary significance such as African swine fever virus, foot and mouth disease virus, Pseudorabies virus and Classical swine fever virus are known to survive for extended periods in plant-based feed ingredients imported into North America. To mitigate risk, high risk ingredients, such as oil seed meals, are stored in designated facilities for extended periods under controlled environmental conditions to minimize viral infectivity prior to use. The results from this study suggest that a storage period of 30-days at a temperature of 23.9^0 C are required to reduce virus infectivity in plant-based feed ingredients such as soybean meal. The outcomes of the study are important, since previous storage periods for feed were based only on mathematical half-life calculations, not controlled studies using live pathogens and representative conditions. We now have for the first time, scientifically sound data based on the use of infectious agents and representative conditions to advise farmers, feed mill operators, federal officials, regulatory and practicing veterinarians, and feed industry leadership on how long and at what temperature to store feed and feed ingredients, to minimize risk. Hopefully, this information will enhance the application and efficacy of Responsible Imports protocols as we collectively work to manage the global risk of feed.

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Scientific Abstract: Viruses of veterinary significance such as African swine fever virus, are known to survive for extended periods in plant-based feed ingredients imported into North America. To mitigate risk, high risk ingredients, such as oil seed meals, are stored in designated facilities for extended periods under controlled environmental conditions to minimize viral infectivity prior to use. While 30 days has become a standard storage period, the required ambient temperature to inactivate viruses during this time is not known. To address the question, 1-metric ton totes of conventional soybean meal were inoculated with PRRSV 144 lineage 1C variant and SVA, stored for 30 days at 23.9^0 C, 15.5^0 C, or 10^0 C, and fed to pigs. Virus infectivity was evaluated through detection of viral RNA in oral fluid samples, along with clinical signs. Results indicated that inactivation of both viruses occurred in soy stored at 23.9^0 C. In contrast, SVA infectivity was observed in soy stored at both 15.5^0 C and 10^0 C, while PRRSV 144 L1C variant infectivity was observed in soy stored at 10^0 C. These results suggest that a storage period of 30-days at a temperature of 23.9^0 C are required to reduce virus infectivity in plant-based feed ingredients such as soybean meal.

Introduction:
North America currently imports plant-based feed ingredients, i.e., soybean meal from countries that are endemically infected with foreign animal diseases viral agents, including porcine epidemic diarrhea virus (PEDV), Seneca virus A (SVA), porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus, pseudorabies virus, foot and mouth disease virus, and African swine fever virus. These viruses all survive in conventional soybean meal for 30 to 37 days, and the T ½ of ASFV in conventional soybean meal, organic soybean meal and soy oil cake is 9.6, 12.9, and 12.4 days, respectively. The North American swine industry has attempted to mitigate this risk through many ways, including mechanical reduction (flushing and sequencing), pelleting, chemical mitigation, and extended storage under controlled environmental conditions. This latter approach has been applied across North America, with Canada developing a national policy-based program and the US swine industry adopting a voluntary program known as "Responsible Imports". However, these protocols are not standardized and have been primarily based on mathematical estimates of half-life, not data derived from controlled challenge studies. The purpose of this study was to determine the required ambient temperature to inactivate two significant viral pathogens of pigs using representative volumes of soybean meal stored in controlled environments for a 30-day period, using natural feeding behavior to determine infectivity The study was based on the hypothesis that virus survival in feed will be negatively impacted by increasing temperature.

Objectives: To determine the necessary ambient temperature required to inactivate Senecavirus A and PRRSV 144 L1C variant in 1-ton totes of conventional soybean meal during a 30-day extended storage period.

Materials & 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 2021-13.

Experimental facilities: The study utilized three facilities: a commercial warehouse for tote preparation and inoculation, a temperature-controlled trailer for the 30-day storage of totes, and the Pipestone Research BSL-2 facility for the assessment of virus infectivity. At the warehouse, a total of 18 1-ton totes of conventional soybean meal were prepared. As previously defined, conventional soybean meal contained a low fat (1-2%) and high protein (46-47%) content. This ingredient was added to new polypropylene bags each with a capacity of 1.74 m^3 (National Bulk Bag, Champlin, MN US), resulting in the 18 totes to be used in the study. This number of totes was based on six totes per temperatures to be tested (23.9^0 C, 15.5^0 C, or 10^0 C) and the six available rooms in the BSL-2 facility. As the experimental unit was the room, a sample size of six rooms per temperature could detect a 75% difference in infection rates with a 95% confidence and 80% power.
Sample preparation and tote inoculation: Viruses selected for inoculation included PRRSV-144 L1C variant and SVA, based on the stability of SVA in soybean meal and the recent emergence of the highly pathogenic PRRSV L1C variant and subsequent industry concern of potential transmission through feed. To simulate a "hot spot" model of feed contamination, 10 mL ice cubes containing a mixture of PRRSV 144 L1C variant and SVA at a total dose of 1 x 10^5 $TCID_{50}$ per virus were prepared. Each virus was diluted in 30 mL of minimum essential medium (MEM, Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 1 x 10^5 $TCID_{50}$/mL per virus and mixed (three viruses for a total of 90 mL) followed by an addition of 210 mL MEM, to bring the total volume to 300 mL. Ice cubes were prepared by freezing 10 mL aliquots of the mixture in 50 mL conical centrifuge tubes (Corning Inc. Corning, NY, USA) at -80^{0}C. The 18 totes were divided into three groups of six, according to the three temperatures to be tested. The first set of totes was inoculated and stored at 23.9^{0}C. To inoculate totes, a solid metal rod (1.2 m in length and 4.45 cm in diameter), was inserted into the lumen of a PVC pipe (1.2 m in length and 5.08 cm in diameter). The combined rod and pipe "instrument" was manually forced into the middle of each tote so as only 0.35 m of the instrument was not covered by feed material. Once the instrument was in the proper place, the internal metal rod was removed, providing a clear path for the ice cube to travel into the interior of the tote,

unimpeded by the presence of meal. Following removal of the rod, the 10 mL ice cube was dropped into the PVC tube, the PVC tube was removed, resulting in the cube being buried by the meal and creation of the "hot spot".

Storage procedure: Immediately following inoculation, each set of six totes were placed into temperature-controlled trailer and stored for 30 days at the designated temperature assigned to each replicate. The trailer was a 2017 14.6 m Hyundai Thermotech Refrigerated Trailer with a Thermoking reefer unit (Sumrall Truck and Trailer Service, Hammond, LA, US). The 23.9^0 C replicate was conducted first, followed by the 15.5^0 C replicate, then the 10^0 C replicate. The trailer was cleaned and disinfected and allowed to sit empty for 30 days between replicates.

Virus infectivity assessment: Following completion of the designated 30-day storage period, each set of six totes were transported to the BSL-2 facility in the refrigerated trailer. This facility had six rooms, each with its own feed bin, allowing for an individual tote to be allocated to a specific bin and be fed to a specific group of pigs. These rooms were organized into separate airspaces using filtration of incoming and outgoing air, with separate shower in/out and Danish entry protocols per room, along with the use of separate clothing, footwear, disposable gloves, and equipment per room to prevent room-to-room cross-contamination. During each replicate, each room in the facility was stocked with 30 six-month-old finishing pigs, originating from a PRRSV and SVA negative farm. This number and size of pigs was chosen based on an estimated average daily feed intake of 2.3 kg/pig. At this rate of consumption, it was estimated that it would require approximately 14 days to consume the entire amount of soybean meal placed in the feed bin. Upon disappearance of the soybean meal, the animals were fed a balanced diet to offset any nutritional deficiencies provided by the soy-only diet. Prior to the experiment, this approach was discussed with the Pipestone IACUC and the Pipestone Nutrition team to anticipate and mange any concerns that could arise from feeding straight soybean meal for 14 days. No concerns were noted. During the 30-day replicate, all six rooms of pigs were assessed for evidence of SVA and PRRSV 144 L1C infection through the collection of oral fluids (one rope per pen, six ropes per room) at day 0 (arrival), 14, and 30 of each replicate. In addition, daily observations were conducted, looking for clinical signs of SVA infection (vesicles and lameness) and signs of PRRSV 144 L1C variant infection (pyrexia, hyperemia, dyspnea, and weight loss). Selected cases of mortality were necropsied, lymphoid tissues collected, and tested by PCR for the presence of SVA and PRRSV RNA.

Environmental monitoring during storage: During each replicate, two temperature and relative humidity (% RH) data loggers (RC-51H waterproof USB temperature data logger, Elitech, San Jose, CA, US) were placed within the trailer to collect environmental data within the trailer during the storage period. Each logger was placed 11.5 cm from the floor, on the wall surface in adjacent corners. One was placed in the rear end of the trailer on the right side, and one was placed in the front of the trailer on the left side. Placement of loggers was consistent for all 3 tests.

Statistical analysis: Due to small sample sizes, a Fisher's exact was used to assess for the significance of the association between tote holding temperature and room-level infection rates overall. A Fisher's exact two-sided test was used when the association was stratified by pathogen, again due to the small sample sizes. The level of significance used for the omnibus comparison was 0.1 and the pairwise temperature comparisons p-values were evaluated against levels of significance calculated using the Benjamini-Hochberg method with a false discovery rate equal to the level of significance (0.1). This latter procedure was employed to statistically enhance the power of the study while conducting multiple comparisons compared to traditional, more conservative adjustments such as Bonferroni's. The Benjamini and Hochberg method ranked the p-values and generated different "cut-off values" based on the false discovery rate and the rank number of the comparison being made. STATA version 16.1 IC statistical software (Stata Corporation, College Station Texas) was used with data stored in Microsoft Excel version 16.56 spreadsheets (Microsoft Corporation, Redmond, WA, US).

Results: Clinical and diagnostic observations by replicate

All oral fluid samples collected from all pens across the three replicates were PCR-negative for SVA and PRRSV RNA on day 0 (arrival of pigs). During the 23.9^0 C replicate, oral fluid samples collected at 14 and 30 days were PCR negative, and vesicles and lameness were not observed. In the 15.5^0 C replicate, SVA RNA was detected in oral fluid samples, and vesicles on the feet and snout with lameness was observed in five of the six rooms. No PRRSV -positive oral fluids were detected and no PRRSV-related clinical signs were observed. In support of these data, SVA RNA were detected in tissue samples from selected clinically affected animals that had died during the study period. In the 10^0 C replicate, SVA RNA in oral fluid samples were detected and clinical signs of SVA were observed in four of six rooms. In addition, PRRSV RNA was detected in oral fluid samples, along with observation of clinical signs of PRRSV in four of six room. In support of these data, SVA RNA and PRRSV 144 L1C variant RNA were detected in tissue samples from selected clinically affected animals that had died during the study period.

Data analysis

The difference in infection at 23.9^0 C versus the other two temperatures was significant at $p = 0.005$. Overall, there was a significant ($p = 0.015$) association between tote holding temperatures (23.9^0 C vs. 15.5^0 C and 23.9^0 C vs. 10^0 C) and detection of either SVA or PRRSV. Further analysis revealed an association between disease presence and temperature of storage when comparing 10°C to 23.9° C and 15.5° C to 23.9° C, as both p-values were below the sequential Benjamini-Hochberg statistic cut-off values, and there was no difference between 10° C and 15.5° C. When the data were stratified by pathogen, there were still significant differences in the holding temperatures and the infectivity of the soy for both pathogens ($p\text{-value}_{SVA}=0.025$; $p\text{-value}_{PRRSV}=0.015$). Furthermore, there were different patterns of significant association that emerged between holding temperatures based on the pathogen. For SVA, the rate of infection did not reach zero for all rooms until the soy was held at 23.9° C. The 23.9° C holding temperature was significantly different from the 15.5° C and 10° C, while the latter two were not different from one another. For PRRSV 144 L1C variant, all the rooms remained uninfected when fed soy held at 15.5° C and 23.9° C. In contrast, 66.7% of the rooms were infected when fed soy held at 10°C. As this sample size was built to detect differences of 75%, there was not enough power to detect a significant difference between 10° C and 15.5° C or 10° C and 23.9° C holding temperatures for PRRSV. Finally, since all rooms tested negative at 15.5° C and 23.9° C, a comparison was not possible.

Temperature and % relative humidity data

Over the course of the 30-day period at a storage temperature of 29.3^0 C, the mean temperature across both loggers was 22.8^0 C with a mean RH of 62.4%. At a storage temperature of 15.5^0 C, the mean temperature across both loggers was 15.3^0 C with a mean RH of 63.4%. Finally, at a storage temperature of 10.0^0 C, the mean temperature across both loggers was 9.5^0 C with a mean RH of 27.5%.

Discussion:

The purpose of this study was to determine the required ambient temperature to inactivate two significant viral pathogens of pigs during the storage of soybean meal for a 30-day period. Under the conditions of this study, we learned that holding feed at higher temperatures, i.e., 23.9^0 C, significantly reduced SVA and PRRSV L1C variant infectivity. We also learned that SVA demonstrated greater survivability than PRRSV, as evidenced by pigs developing disease when fed contaminated soy held at 15.5° C, while the PRRSV infectivity was neutralized when contaminated soy was held at 15.5° C, but not 10.0^0 C.

The strengths of this study were its practical approach and the use of a rigorous experimental design to answer the specific research question. We also used SVA, a virus known to be very stable in feed as well as a validated surrogate for FMDV, in combination with an ingredient (conventional soybean meal) know to be very protective to multiple viruses, to generate a “worst case scenario” to best test the efficacy of the protocol under BSL-2 conditions.

In contrast, a limitation to this study is the sample size. The sample size used could only detect ≥ 75% difference between holding temperatures with 95% confidence and with 80% error and ≥ 65% difference at a 90% confidence infectivity. Further limitations included the inability to use ASFV; however, we were able to use SVA, a virus known to be very stable in feed.

In closing, the outcomes of the study are important, since previous storage periods for feed were based only on mathematical half-life calculations, not controlled studies using live pathogens and representative conditions. We now have for the first time, scientifically sound data based on the use of infectious agents and representative conditions to advise farmers, feed mill operators, federal officials, regulatory and practicing veterinarians, and feed industry leadership on how long and at what temperature to store feed and feed ingredients, to minimize risk. Hopefully, this information will enhance the application and efficacy of Responsible Imports protocols as we collectively work to manage the global risk of feed.

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