RESEARCH REPORT
SWINE HEALTH

Title: PEDv Survivability in Manure-Amended Soil and Evaluation of Lime Application to Soil as a PEDV Biosecurity Measure – **NPB #14-269**

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Industry Summary

This project was conducted in an effort to determine the transmission risk for PEDv in stored manure and manure-amended soil. Specific objectives of this project were to: 1) determine the survivability of the PED virus over time in two common soils at two moisture regimes treated with PEDv-positive swine slurry and held at temperatures representing three climates (southern Minnesota, northern Missouri, and central Oklahoma); and 2) determine the impact of lime application to manure on PEDv survivability.

PEDv Survivability in Soil

The manure+soil incubation experiment investigated how PEDv survived in two manure-amended soils (silty clay loam and loamy fine sand) at two initial moisture conditions (10 and 30% water holding capacity) and under three different winter climate conditions (southern Minnesota, northern Missouri, and central Oklahoma). PEDv-positive manure slurry was divided into two 3-L quantities of manure. Quick lime (30 g) was added to one 3-L portion of manure (equivalent to 80 lbs. quick lime per 1000 gallons of slurry) to achieve a final pH of 12. After 24 h, 10 mL of slurry from the two sources (un-limed or limed) were added to 50 mL conical tubes containing 30 g of soil under the two soil types and two moisture conditions. Tubes were loosely capped and placed into one of three incubators operated independently throughout the trial to simulate soil temperatures between November 1 and May 1 at one of three geographic locations: southern Minnesota, northern Missouri, and central Oklahoma. A set of four replicated samples for each treatment combination was removed on days 0, 30, 60, 90, 120 and 150 of the incubation and immediately transferred to a -80°C freezer for storage. After the 150-day incubation, RNA was extracted from each soil sample using the MO BIO RNA PowerSoil kit. PEDv was detected in samples by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) using a standard prepared from extracted PEDv RNA.

Based upon other studies conducted concurrently, only samples collected on days 0 and 30 were analyzed by qPCR. The other manure+soil samples (days 60, 90, 120, and 150) are still available for analysis, but were not analyzed because they likely do not contain any infectious PEDv. Soil receiving un-limed manure had a pH of 7.45 to 7.96 immediately following application of manure (day 0) and 8.15 to 8.74 at day 30. The pH of soil receiving limed manure was 8.60 to 9.27 at day 0 and 8.15 to 8.74 at day 30. Results revealed that abundance of PEDV RNA decreased immediately to background levels following manure addition to soil, regardless of

whether the manure was limed or un-limed. No differences in abundance of PEDv RNA were observed based on soil type, initial soil moisture, or incubation condition. From this data, it was concluded that a soil pH of 7.5 or greater is sufficient to inactivate the PED virus. When utilizing manure from a PEDv-positive site as a soil amendment, the pH of the receiving soil should be tested. If the pH is 7.5 or greater, the soil is not expected to serve as a vector for the virus. Soils with a pH below 7.5 may still serve as a vector for the virus and lime addition to the soil or manure is recommended to ensure that the virus does not survive in the soil environment.

Impact of Lime Application on PEDv Survivability

Three separate studies were conducted under this objective. In the first study, PEDv-positive manure (RT-qPCR Ct value of 25) was collected from the pit of a commercial farm. Three samples (250-mL) at initial pH 7.54 were placed into glass beakers and quick lime was added in 0.25-g increments until pH 12 was achieved. This experiment revealed that 2.5 g of quick lime per 250 mL of slurry achieved a final manure pH of 11.9. This equates to 83.5 lbs of quick lime per 1000 gallons of manure at an initial pH of 7.54.

A second study was conducted to determine how quickly the PED virus is inactivated when exposed to manure with pH 12. Manure slurry from a commercial farm (RT-qPCR Ct value of 25 and pH 7.54) was treated with quick lime to achieve pH 12 (n=3) and compared to non-limed slurry (n=3). Both treatments were sampled at time = 0, 1, 12, and 24 h and analyzed for the presence of the PED virus RNA. Results revealed that limed manure at t = 1, 12, and 24 h no longer contained detectable PED virus.

A third study was then conducted to determine if a lower pH could be effective at inactivating the virus. Three treatments were compared: manure limed to achieve pH 10, manure limed to achieve pH 12, and manure with no lime addition. Three replicates were utilized per treatment. Samples (250-mL) of pooled PEDv-positive manure (RT-qPCR Ct value of 23) collected from swine that had been experimentally infected with PEDv strain CO13 was distributed among nine beakers (n=3 per treatment). Specimens were sampled at time = 0 and then lime was added to three beakers to achieve pH 10 and to three additional beakers to achieve pH 12. Sub-samples (10 mL) were collected from each beaker at 1 and 10 h following lime addition, neutralized, and stored at -80°C. PCR analysis of samples revealed that PEDv RNA was not detected in any of the limed samples (pH 10 or 12 with exposure times of 1 or 12 h), while untreated manure contained detectable virus.

To confirm that conditions yielding a PCR-negative result truly inactivated the PED virus and rendered the manure non-infectious, a live pig bioassay was conducted with the limed and non-limed manure slurry samples. Fifteen pigs, approximately 21 days old, were sourced from a high-health facility with dams testing negative for PEDv antibodies and virus by PCR. Piglets were tested for PEDv upon arrival and confirmed negative. Piglets were randomly assigned to individual housing in BSL-2 animal rooms: control (3 piglets), pH 10 (6 piglets), and pH 12 (6 piglets), and allowed to acclimate for three days. Each pig was then administered a 10 mL oral gavage of manure slurry: three piglets in the control room received one of the three un-limed slurry samples; six pigs in the pH 10 room received one of the six limed (pH 10) slurry samples (three limed for 1 h and three limed for 10 h); and six pigs in the pH 12 room received one of the six limed (pH 12) slurry samples (three limed for 1 h and three limed for 10 h). Pigs were monitored for fecal shedding of PEDv for four days until control animals began to demonstrate clinical signs of PEDv infection, at which time all piglets were humanely euthanized. Fecal swabs, and duodenum, ileum, jejunum, and cecum tissue samples were collected from each animal and fixed in formalin. All fecal and tissue samples were analyzed for the presence of detectable PED virus by immunohistochemistry and PCR. All pigs receiving limed manure (pH 10 or 12 maintained for 1 or 10 h) demonstrated no clinical signs of PEDv infection while control pigs (un-limed treatment) all tested positive for PEDv infection.

Conclusions garnered from this project include:

1. **Lime addition to manure to achieve pH 10 for at least 1 h is sufficient to inactivate the PED virus.**
2. **For stored manure slurry at an initial pH of 7.5 or greater, addition of 50 pounds of quick lime per 1000 gallons of manure slurry is required to achieve a final pH of 10.**

3. **Addition of lime to manure in a storage pit is not recommended due to significant precipitation of solids resulting from the lime addition, which can create a thick sludge that is difficult to remove from the storage. Likewise, ammonia volatilizes more rapidly as pH increases so liming of stored manure can create significant odor and a potentially harmful concentration of ammonia gas. Liming of manure in a tank wagon prior to transport of manure to a land application site is the recommended alternative.**
4. **Application of PEDv-positive swine manure to soil with pH 7.5 or greater does not present a risk for survival of the virus in the soil.**

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Abstract

There is concern that manure infected with the porcine epidemic diarrhea virus (PEDv) could serve as a vector for reinfection or disease transmission if the virus survives in stored manure or manure-amended soil. In regions of the U.S. where winter soil temperatures commonly remain near freezing and precipitation is sufficient to maintain moderate to high soil moisture content in the top 10 cm of the soil, the common practice of applying manure to soil following fall crop removal presents a potentially ideal environment for the virus to remain infectious in the soil. This project was designed to determine the transmission risk for PEDv in stored manure and manure-amended soil. Specific objectives of this project were to: 1) determine the survivability of the PED virus over time in two common soils at two moisture regimes treated with PEDv-positive swine slurry and held at temperatures representing three climates (southern Minnesota, northern Missouri, and central Oklahoma); and 2) determine the impact of lime application to manure on PEDv survivability.

Introduction

Research has shown that the porcine epidemic diarrhea virus (PEDv) survives well in cool, moist environments. In regions of the U.S. where winter soil temperatures commonly remain near freezing and precipitation is sufficient to maintain moderate to high soil moisture content in the top 10 cm of the soil, the common practice of applying manure to soil following fall crop removal presents a potentially ideal environment for the virus to remain infectious in the soil. If the virus remains viable in the soil, the potential exists for the PED virus to be transported from the field by equipment during spring fieldwork activities. Currently, the ability of the PED virus to remain viable in manure-amended soil is unknown. Therefore, this project was designed to assess the survivability of PEDv in manure-amended soil during winter months in three geographic regions of the U.S. and determine the impact of utilizing lime as a treatment for inactivating the PED virus in stored manure.

Objectives

This project was conducted to achieve the following research objectives:

1. Determine survivability of the PED virus over time in two common soils treated with PEDv-positive swine slurry at two moisture regimes and in three climates.
2. Determine the effects of manure pH adjustment with lime at pH 10 or 12 for exposure times of 1, 12, or 24 h on PED virus survivability and infectivity in manure.
3. Determine the effects of manure pH adjustment by lime application on ammonia volatilization and manure nitrogen concentration.

Materials & Methods

Soil Survivability Study

Manure Preparation. Swine manure slurry (RT-PCR Ct value of 26.46) was collected from the shallow pit of a PEDv-positive commercial sow operation in central Nebraska and screened by the University of Nebraska (UNL) Veterinary Diagnostic Clinic (VDC) for PEDV and other enteric viruses and bacteria that could confound the bioassay.

Soil preparation. Soil representing two common soil types (silty clay loam and loamy fine sand) were collected and analyzed for water holding capacity, pH, soluble salts, organic matter, nitrate-nitrogen, phosphorus, potassium, calcium, magnesium, sodium, sum of cations (CEC), and soil classification (% clay, silt, and loam). Soils were prepared by passing through a No. 10 sieve to achieve a uniform particle size distribution in all tests. Each soil type was separated into two subsets and distilled water was added to each subset to achieve either 10 or 30% water holding capacity (WHC). Thirty grams of soil for each soil type and WHC combination was placed into 50-mL conical tubes and 10 mL of manure (limed or un-limed) was applied to the soil in each tube. Tubes were loosely capped and placed into one of three incubators operated independently throughout the trial according to the soil incubation profiles described previously.

Soil Incubation. Multi-year soil temperature data sets were acquired for three climate regions – southern Minnesota, northern Missouri, and southeast Oklahoma – and were used to develop mean soil temperature curves for simulating 180 days of soil temperature for each region from approximately October 1 through March 31 (or until planting temperature is achieved in the spring). A daily schedule of incubator temperature adjustments was then developed to represent each of the three climates. In addition to daily temperature adjustments, freeze-thaw and thaw-freeze cycles were incorporated into each climate treatment to represent temporal changes in the top 0 to 10 cm of soil during the winter months observed in historical data. The total number of freeze-thaw/thaw-freeze cycles was 20, 13, and 26 for Minnesota, Missouri, and Oklahoma, respectively. Four replicated samples for each treatment combination were removed from the incubators on days 0, 30, 60, 90, 120 and 150 and transferred to a freezer for storage at -80°C.

Soil Analysis. Each tube of soil was processed by [steps in preparing each tube for RNA extraction]. The MOBIO RNA PowerSoil Kit was used to extract ribonucleic acid (RNA) from each sample and PEDv RNA was detected by reverse transcription and quantitative polymerase chain reaction (qPCR) using a standard prepared from extracted PEDv RNA.

Manure Liming Study

Manure Collection. Swine manure slurry (RT-PCR Ct value of 26.46) was collected from the shallow pit of a PEDv-positive commercial sow operation in central Nebraska and screened by the University of Nebraska (UNL) Veterinary Diagnostic Clinic (VDC) for PEDV and other enteric viruses and bacteria that could confound the bioassay.

Liming Exposure Time. To determine how quickly PEDv could be activated in limed manure slurry at pH 12, 250 mL of manure was placed into each of six glass beakers and 5 g of quick lime was added to each of the three of the beakers to achieve pH 12. Three beakers were left untreated to serve as controls. For both treatments (limed and un-limed), 40 mL of manure was collected 1, 12 and 24 h post-treatment application and analyzed for the presence of PED virus RNA.

Liming pH. To determine whether PEDv could be inactivated in limed manure slurry at pH 10, 250 mL of PEDV positive manure (RT-PCR Ct value of 23) collected from swine that had been experimentally infected with PEDV strain CO13 was placed into each of nine glass beakers. Quick lime was added to each of three beakers to achieve pH 12, to each of three other beakers to achieve pH 10, and the remaining three beakers were left untreated to serve as controls. Samples (10 mL) from each beaker were collected at 1 and 10 h post-treatment application, neutralized to pH 7 with hydrochloric acid and transferred to a freezer for storage at -80°C.

Live Swine Bioassay. To confirm that PCR-negative samples were truly non-infectious, a live pig bioassay was conducted with samples from the "Liming pH" experiment. Fifteen 21-d-old pigs were sourced from a high-health facility whose dams tested negative for PEDv antibodies and virus by PCR. Piglets were tested for PEDv upon arrival and confirmed negative. Piglets were randomly assigned to individual housing one of three BSL-2 animal rooms: Room 1 (3 piglets), Room 2 (6 piglets), and Room 3 (6 piglets), and allowed to acclimate for three days. Each pig was then administered a 10 mL oral gavage of manure slurry: Room 1 pigs each received one of the three un-limed slurry samples (control); Room 2 pigs each received one of the six limed slurry samples treated to pH 10 for 1 or 10 h; and Room 3 pigs each received one of the six limed slurry samples treated to pH 12 for 1 or 10 h. Pigs were monitored for fecal shedding of PEDv for four days until control animals began to demonstrate clinical signs of PEDv infection, at which time all piglets were humanely euthanized. Fecal swabs, and duodenum, ileum, jejunum, and cecum tissue samples were collected from each animal and fixed in formalin for analysis by RT-PCR and immunohistochemistry for the presence of PEDv.

Manure Ammonia Loss Determination. To estimate ammonia loss occurring when manure in a deep pit or slurry tank wagon is treated with lime to pH 12, simulations of these two storage types were created in a laboratory setting. A 250-mL volume of manure collected from a commercial sow operation was placed into each of three glass beakers to simulate deep pit manure storage, while 20 mL of manure was placed into each of three 50-mL conical tubes to simulate manure transportation in a tank wagon. Each beaker was treated with 5 g of quick lime to achieve pH 12. Conical tubes were each treated with 0.4 g of quick lime to achieve pH 12 and loosely capped. Sub-samples of each beaker and tube were collected over time following brief agitation, neutralized to pH 7 with hydrochloric acid, and analyzed for ammonia concentration (DU 800 Spectrophotometer, Beckman Coulter, Indianapolis, IN).

Results

Objective 1. Determine survivability of the PED virus over time in two common soils treated with PEDv-positive swine slurry at two moisture regimes and in three climates.

Based upon the outcomes of manure liming experiments conducted during the six-month-long soil incubation experiment, only limed and un-limed manure+soil samples collected on days 0 and 30 were analyzed by RT-qPCR. The other manure+soil samples (days 60, 90, 120, and 150) are still available for analysis, but were not analyzed because they likely do not contain any infectious PEDv. Figure 1 summarizes the RT-qPCR results for the soil samples collected on days 0 and 30.

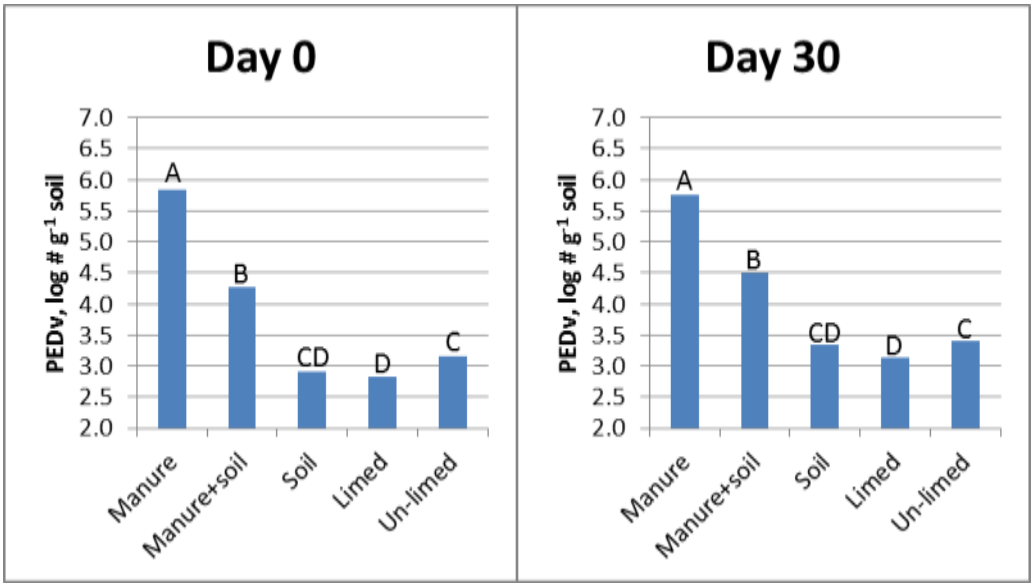

Figure 1. Log abundance of PED virus on days 0 and 30 of the manure+soil incubation study determined by RT-qPCR. Differences between samples for a particular day are designated by different letters above the bar.

The 'Soil' sample is a negative control (contains no PEDv) and represents the limit of detection for the assay based upon amount of soil extracted and fraction of extract used in the RT-qPCR. The 'Manure+soil' sample was an artificial mixture of soil and manure RNA extracted separately (3:1 ratio in the mix) and represents the expected RT-qPCR result for the manure+soil mixture.

No differences in PEDv abundance were detected on day 0 or 30 when initial soil moisture (10% vs. 30% water holding capacity), incubation condition (MN vs. MO vs. OK), or soil type (silty clay loam and loamy fine sand) was varied.

For the soils in this experiment, the quantity of PEDv RNA in limed or un-limed manure decreased immediately to background levels following manure addition to soil (i.e. limed and un-limed manure PEDv RNA quantity did not differ from the background soil).

Objective 2. Determine the effects of manure pH adjustment with lime to pH 10 or 12 for exposure times of 1, 12, or 24 h on PED virus survivability and infectivity in manure.

The results of the "Liming Exposure Time" experiment revealed that manure treated with quick lime to pH 12 no longer contained detectable PED viral RNA at 1, 12 and 24 h. The "Liming pH" experiment revealed that samples treated with quick lime to achieve either pH 10 or 12 for 1 or 10 h did not contain detectable PED viral RNA while untreated manure (0 h) contained detectable virus. The "Live Swine Bioassay" experiment yielded negative immunohistochemistry and RT-qPCR results for tissue and fecal samples, respectively, collected from pigs receiving pH 10 or 12 manure treatments. Conversely, all pigs receiving un-limed manure (control animals) tested positive for PEDv infection. Immunohistochemistry results from control group piglets revealed the presence of PED virus antigen in gastrointestinal tract tissue sections (Figure 2).

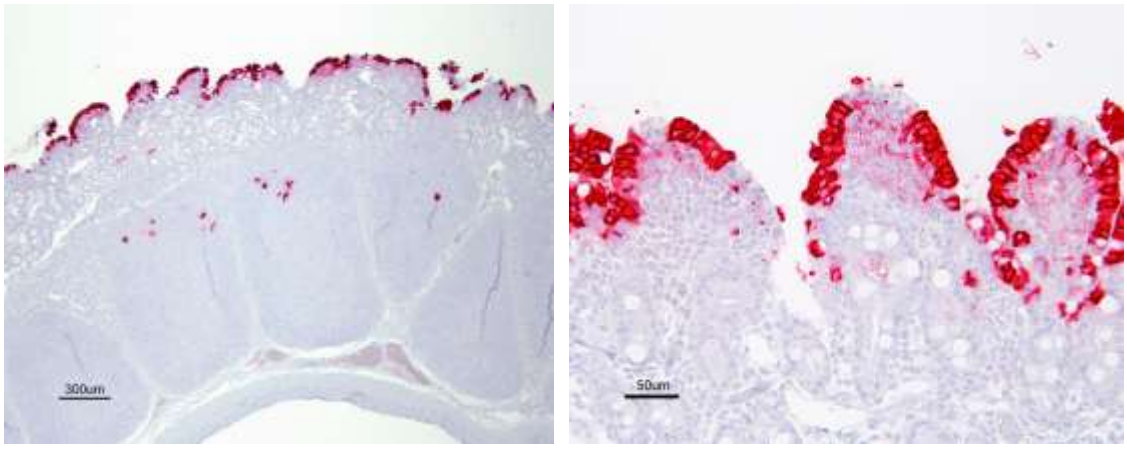

Figure 2. Immunohistochemistry staining of PED virus antigen using PEDV specific antibodies (stained red) in tissue sections from the ileum and jejunum of a PEDV-positive piglet treated with non-limed manure.

Objective 3. Determine the effects of manure pH adjustment by lime application on ammonia volatilization and manure nitrogen concentration.

Initial ammonia concentration of untreated manure averaged 1860 ppm among three replications of the analysis. Following lime addition to the manure samples, nitrification progressed rapidly in all treatments. The deep pit simulation treatments experienced rapid ammonia loss during the first 3 h with continued steady losses totaling 85% through 24 h. The manure tank wagon simulation treatments experienced rapid ammonia loss for the short period (1 h) monitored to represent potential travel time of the tank wagon from filling at the barn to application at the field, revealing a total reduction in manure ammonia concentration of 20%.

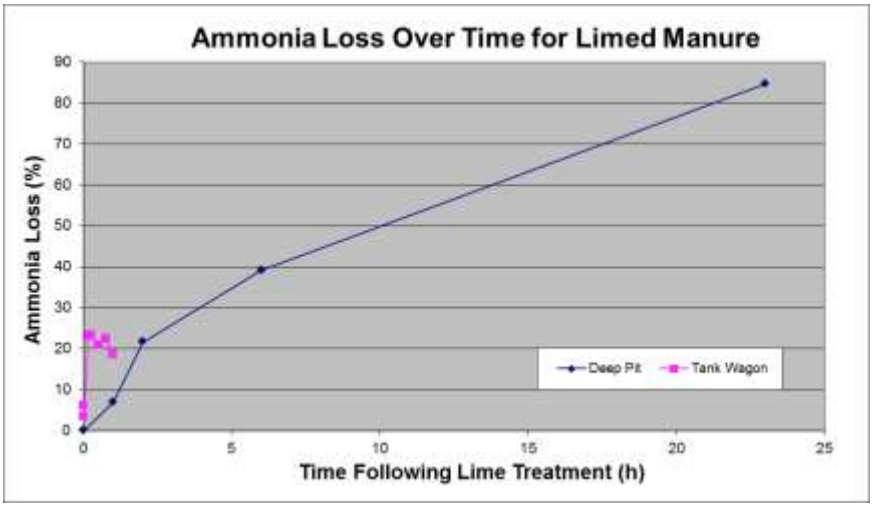

Figure 2. Cumulative ammonia loss over time following lime addition to simulated deep pit manure storage and manure tank wagon

Discussion

Important conclusions and recommendations resulting from this project are:

1. Lime treatment of manure continuing for more than two hours in an open manure storage will likely reduce the nutrient value of the manure considerably and potentially yield dangerously high ammonia concentrations in and around the storage environment. Lime treatment of deep pit manure storages is not recommended!
2. Lime addition to manure to achieve pH 10 for at least 1 h is sufficient to inactivate the PED virus. Approximately 50 pounds of quick lime per 1000 gallons of manure slurry is required to achieve this pH with an initial slurry pH of at least 7.5.
3. Application of PEDv-positive swine manure to soil with pH 7.5 or greater does not present a risk for survival of the virus in the soil.