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Alkaline stabilization of manure slurry inactivates porcine epidemic diarrhea virus

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Summary

Hydrated lime manure treatment was evaluated to determine porcine epidemic diarrhea virus (PEDV) susceptibility to alkaline stabilization. At pH 10, PEDV decreased (quantitative polymerase chain reaction) and lost infectivity (swine bioassay). Although ammonium decreased above pH 9 (up to 25%), alkaline stabilization managed to control potential infection from manure sources.

Keywords: swine, manure, porcine epidemic diarrhea, pH, hydrated lime

Resumen – La estabilización alcalina del estiércol líquido desactiva al virus de la diarrea epidémica porcina

Se evaluó el tratamiento del estiércol con cal hidratada para determinar la susceptibilidad del virus de la diarrea epidémica porcina (PEDV por sus siglas en inglés) a la estabilización alcalina.

A un pH de 10, el PEDV disminuyó (reacción en cadena de la polimerasa cuantitativa) y perdió infectividad (bioensayo porcino). Aunque el amonio disminuyó arriba de un pH de 9 (hasta 25%), la estabilización alcalina logró controlar la infección potencial de las fuentes de estiércol.

Résumé – Inactivation du virus de la diarrhée épidémique porcine par stabilisation alcaline du purin

Un traitement du purin à l'aide de chaux hydratée a été évalué afin de déterminer la sensibilité du virus de la diarrhée épidémique porcine (VDEP) à une stabilisation alcaline. À une valeur de pH de 10 on nota une diminution du VDEP (réaction d'amplification en chaîne par la polymérase quantitative) et une perte d'infectivité (bio-essai sur des porcs). Bien que l'ammonium diminuait à pH au-dessus de 9 (jusqu'à 25%), la stabilisation alcaline a permis de limiter l'infection potentielle à partir du purin.

The emergence of the porcine epidemic diarrhea virus (PEDV) in the United States in 2013 resulted in billions of dollars in annual losses in the US swine industry.[1,2] Infection with PEDV causes severe diarrhea and vomiting in swine, spreads rapidly through ingestion of infected manure, and in naïve herds produces nearly 100% mortality in piglets less than 1 week old. Although the virus persists in feces for several days and may transport several miles from infected production sites as bioaerosol,[3,4] recent research indicates that management strategies can limit the virus' spread between production sites on transportation equipment.[5] However, concerns about virus persistence in various types of manure storage (ie, deep pit, lagoon, or slurry tank) remain a major barrier to proper manure management.

Because swine manure slurry is a valuable source of nitrogen and phosphorus, manure typically is utilized in agricultural fields for crop production. Proper manure handling and application practices are necessary to control the risk of pathogen re-infection at affected production sites, or infecting new sites through virus-contaminated manure-handling equipment. A variety of treatment options have been proposed and evaluated for their capacity to inactivate viruses in swine manure slurry.[6] Hydrated lime [Ca(OH)$_2$] has been demonstrated to inactivate porcine enterovirus types 2 and 3,[7] and alkaline stabilization is an approved treatment for septage prior to land application when a pH of 12 is maintained for at least 30 minutes.[8] However, increasing manure slurry pH may decrease its value as a fertilizer, since ammonia losses through volatilization would be enhanced. It was hypothesized that alkaline stabilization of manure would decrease infectious PEDV in swine production and in manure-handling systems. Laboratory studies were conducted to assess the abundance and survival of PEDV in stored swine manure slurry treated with hydrated lime and to quantify potential ammonia volatilization losses during hydrated lime treatment.

Materials and methods

The experimental protocol was approved by the University of Nebraska-Lincoln Institutional Animal Care and Use Committee prior to the initiation of any research activity.

Manure for the first pH incubation study (conducted in 2015) was collected from swine that had been experimentally infected with PEDV strain CO/13 at the Life Sciences Annex at the University of Nebraska-Lincoln School of Veterinary Medicine and Biomedical Sciences (UNL VMBS).[9] Manure for the second study was collected in 2016 at a commercial breed-to-wean operation in south central Nebraska. At the commercial location, freshly excreted swine manure solids were collected into sterile sample containers from the floor surfaces in

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This article is available online at http://www.aasv.org/shap.html.

four separate farrowing room sites showing clinical signs of suspected porcine epidemic diarrhea, and transported on ice to the University of Nebraska-Lincoln. Prior to use in incubation studies, manure samples were confirmed as PEDV-positive using a reverse transcriptase polymerase chain reaction protocol (RT-qPCR). The quantification cycle (Cq) value for these manure sources was 23, equivalent to approximately 10^5 virus genomes per PCR reaction.

The first alkaline stabilization incubation had triplicate manure slurries consisting of fresh manure (UNL VMBS) and deionized water (final composition: 18.5% solids content, 38.4% "volatile" by combustion loss at 550°C). The three slurries were mixed and sampled prior to any treatment (time = 0 hours, no hydrated lime added). Each slurry was then distributed (250 mL) into two glass beakers (six total). Each pair received 1.5 g and 2.5 g of hydrated lime per L to achieve a final pH of 10 or 12, respectively. Aliquots (10 mL) were collected from each beaker at 1 and 12 hours following hydrated lime addition, immediately neutralized with 10 mM HCl, and frozen at -80°C for subsequent analysis.

In the second alkaline stabilization incubation, manure samples were collected at four replicate sites at the commercial swine operation. To better mimic the typical consistency of stored manure slurry, each manure sample was mixed in equal portion with deionized water (1 kg manure:1 L H_2O) prior to treatment (final composition: 21.6% total solids, 80.2% "volatile" by combustion loss). Each 250-mL replicate of slurry received stepwise (0.25 g) additions of hydrated lime with continuous stirring to gradually increase manure slurry pH to 12. After each addition of hydrated lime, pH was determined (FiveEasy Plus; Metter-Toledo AG, 8603 Schwerzenbach, Switzerland) and duplicate 2-mL samples of each manure slurry were collected, immediately neutralized (10 mM HCl), and stored at -80°C for subsequent PEDV RNA copy enumeration and infectivity in a pig bioassay.

A PCR approach was used to quantify PEDV genomes in manure samples. The RNA in each manure slurry sample was extracted using TRIzol reagent following manufacturer's suggested protocol for biological liquids and hard to lyse samples (Life Technologies, Carlsbad, California). Bead mill homogenization using 0.1-mm glass beads in an Omni Bead Ruptor (Omni International, Kennesaw, Georgia) at 4.5 meters per second for 45 seconds was included in the protocol to aid in cell lysis. An RT-PCR product was generated from RNA extracted from reference PEDV (CO/13) using primers and conditions as previously described.[10] Run-off transcripts were generated from the T7 promoter on the PEDV forward primer using the MEGAshortscript T7 kit (Invitrogen, Carlsbad, California). Transcripts were quantified by RiboGreen fluorometry (Turner BioSystems, Sunnydale, California), and then 10-fold serial dilutions of the transcripts were prepared at concentrations ranging from 1×10^1 to 1×10^6 copies of PEDV (as RNA targets) per µL for subsequent RT-qPCR. Quantification of PEDV genomes in the purified manure slurry RNA extracts was accomplished using an Applied Biosystems StepOnePlus thermal cycler (ThermoFisher Scientific, Waltham, Massachusetts), primers, probes, and amplification conditions as previously described,[10] with the exception that internal PCR probe contained both 3' Iowa Black fluorescence quencher and an internal ZEN quencher (Integrated DNA Technologies, Coralville, Iowa) located nine bases from the 5' end. Briefly, one step RT-qPCR was carried out in a 20-µL reaction containing 1 µL of RNA extract or RNA standard, 0.1 µL of both PEDV forward and reverse primer, 0.25 µL of PEDV internal PCR probe, 12.5 µL of QIAGEN Quanti-Tect Probe Taq enzyme mix, 0.25 µL QIA-GEN QuantiTect Probe reverse transcriptase mix, and 5.8 µL of water. Thermal cycler conditions: initial reverse transcription at 50°C for 30 minutes, followed by initial denaturation at 95°C for 15 minutes, 40 cycles of denaturation at 94°C for 15 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 30 seconds. All RT-qPCR runs had reported efficiencies > 80% and R^2 > 0.997.

Two swine bioassays were conducted with the alkaline stabilized and non-stabilized PEDV-infected manure slurry samples in order to relate RT-qPCR results with disease infectivity (Figure 1). For the first study, 15 pigs (approximately 21 days old) were sourced from a high-health facility whose dams tested negative for PEDV antibodies and virus by PCR. Pigs were tested for PEDV upon arrival and confirmed negative by fecal swab RT-qPCR. Pigs were each randomly assigned to individual housing in one of three BSL-2 animal rooms at the University of Nebraska-Lincoln Life Sciences Annex, grouped as follows, and allowed to acclimate for 3 days: control (three pigs), pH 10 manure (six pigs), and pH 12 manure (six pigs). Each pig was then administered a 10-mL oral gavage of diluted manure slurry from the first alkaline stabilization incubation (1 part manure slurry: 9 parts sterile buffer): three pigs in the control room each 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 hour and three limed for 12 hours); and six pigs in the pH 12 room received one of the six limed (pH 12) slurry samples (three limed for 1 hour and three limed for 12 hours). Pigs were monitored for fecal shedding of PEDV for 4 days until control animals began to demonstrate clinical signs of PEDV infection, at which time all pigs were humanely euthanized. Fecal swabs and ileum, jejunum, and mesenteric lymph node tissue samples were collected from each animal and fixed in formalin. Fecal and tissue samples were analyzed for the presence of PEDV by immunohistochemistry(IHC)[9] and RT-PCR (Cq only).

The second bioassay used a similar design, including pig source, history, age, housing, inoculation, and processing to assess PEDV infectivity in the various samples from the second incubation study. Manure slurry samples were selected from three of the manure slurries at points where pH was closest to 7, 8, 9, 10, or 11. Fifteen pigs were housed in three rooms (five per room) with one animal in each room receiving one of the five pH-diluted manure slurries by oral gavage. Pigs were monitored for signs of disease for a week prior to euthanasia. Fecal swabs and tissue samples were collected and tested for the presence of PEDV.

A third manure slurry incubation was conducted to assess changes in nitrogen content, since alkaline stabilization may enhance ammonia volatilization from treated manure during simulated storage in a deep pit or transport in a manure tank wagon. Fresh manure samples were collected from three replicate locations at the commercial site, diluted to create manure slurry (1 kg manure:1 L H_2O), and distributed into ten 250-mL bottles. Five bottles were each randomly assigned to one of two treatments: simulated storage in a manure pit (PIT) or simulated transport in a manure tank wagon (TANK), and hydrated lime additions were randomly applied to each manure slurry (n = 3) within PIT or TANK

blocks to achieve one of five pH endpoints: 8.0, 8.5, 9.0, 9.5 and 10.0. To mimic deep pit storage at a swine production site, PIT bottles were left uncapped while the trial was conducted. To mimic storage in a tank, the TANK bottles were tightly capped during the experiment. PIT samples (1 mL) were sampled initially and 24 hours following hydrated lime application (simulated overnight treatment). Samples (1 mL) from the TANK block were collected initially and 2 hours following hydrated-lime application (simulated short-term treatment). All samples were acidified with 20 μL of 10% sulfuric acid to adjust the pH to < 3 and refrigerated until analysis for ammonium using the Phenate method.[11]

ANOVA (SAS version 9.2, SAS Institute, Cary, North Carolina) was used to analyze log-transformed PEDV abundance in the first two manure alkaline stabilization incubations and to analyze ammonium percentage increase or decrease ($1\text{-}C_{final} \div C_{initial}$) in the third manure slurry incubation. For the first incubation, five treatments were compared (control, pH 10 for 1 hour, pH 12 for 1 hour, pH 10 for 12 hours, and pH 12 for 12 hours) with treatment as the main effect comparing log PEDV. In the second incubation, ANOVA was conducted using target pH (7, 8, 9, 10, and 11) as the main effect, comparing log PEDV. For the third incubation, manure storage and manure pH were the main effects, comparing the ammonium percentage increase or decrease.

Results

In the first manure slurry incubation, RT-qPCR analysis of samples detected PEDV RNA sequences in all treatments (hydrated lime or untreated) except at pH 12 after a 12-hour incubation (Table 1). A clear trend for lower PEDV abundance with hydrated-lime addition (pH 10 versus 12) and with increased hydrated-lime exposure time (1 versus 12 hours) was observed. In the swine bioassay, pigs receiving limed manure treatments (pH 10 or 12 incubated for 1 or 12 hours) via oral gavage displayed none of the clinical signs of PEDV infection (eg, diarrhea, dehydration, or vomiting) and did not shed PEDV in the feces (as determined by PCR). All control pigs (n = 3) receiving un-limed manure displayed clinical signs of disease, tested positive for PEDV infection via IHC, and shed PEDV in the feces (ie, had a low Cq by RT-PCR).

In the second manure slurry incubation, stepwise addition of hydrated lime gradually increased the pH of the manure slurries (Figure 2). Quantitative PCR analysis of samples revealed a rapid decline in the number of PEDV copies above pH 10, but no change in the abundance of PEDV targets below pH 10 (10^9 PEDV targets per gram of manure slurry). Swine bioassay results on a subset of those samples were consistent with RT-qPCR results: IHC and RT-PCR detections of PEDV were observed only in pigs exposed to manure slurry when the pH was less than 10 (Table 2).

For the final manure slurry incubation, initial ammonium concentrations varied considerably between the three replicate locations at the commercial site (0.90 ± 0.06, 1.89 ± 0.17, and 2.49 ± 0.24 g NH_4^+ per L). Prior to statistical analysis, final concentrations were normalized to initial concentration for each manure slurry container yielding a percentage increase or decrease ($1\text{-}C_{final} \div C_{initial}$). Of the two main effects (manure storage and manure pH) and their interaction term, only manure pH proved to be significant ($P < .05$). During manure storage, the average ammonium content increased by 6.6%. The largest differences in manure slurry ammonium content were found between low pH (8, 8.5, and 9) and high pH (9.5 and 10) manure samples ($P < .01$). Ammonium in the low pH group increased an average of 15.7% ± 3.9% relative to initial concentrations. In comparison, ammonium in the high pH group decreased by 7.1% ± 3.5%.

Figure 1: For the swine bioassay, pigs were randomly assigned to multiple rooms and housed in individual crates. The pigs were administered diluted, PEDV-positive manure slurry (untreated and treated with hydrated lime) and monitored for several days for signs of disease (including PEDV-specific PCR of fecal swabs). After euthanasia, additional gastrointestinal tissue samples were collected for PCR and immunohistochemistry tests. PEDV = porcine epidemic diarrhea virus; PCR = polymerase chain reaction.
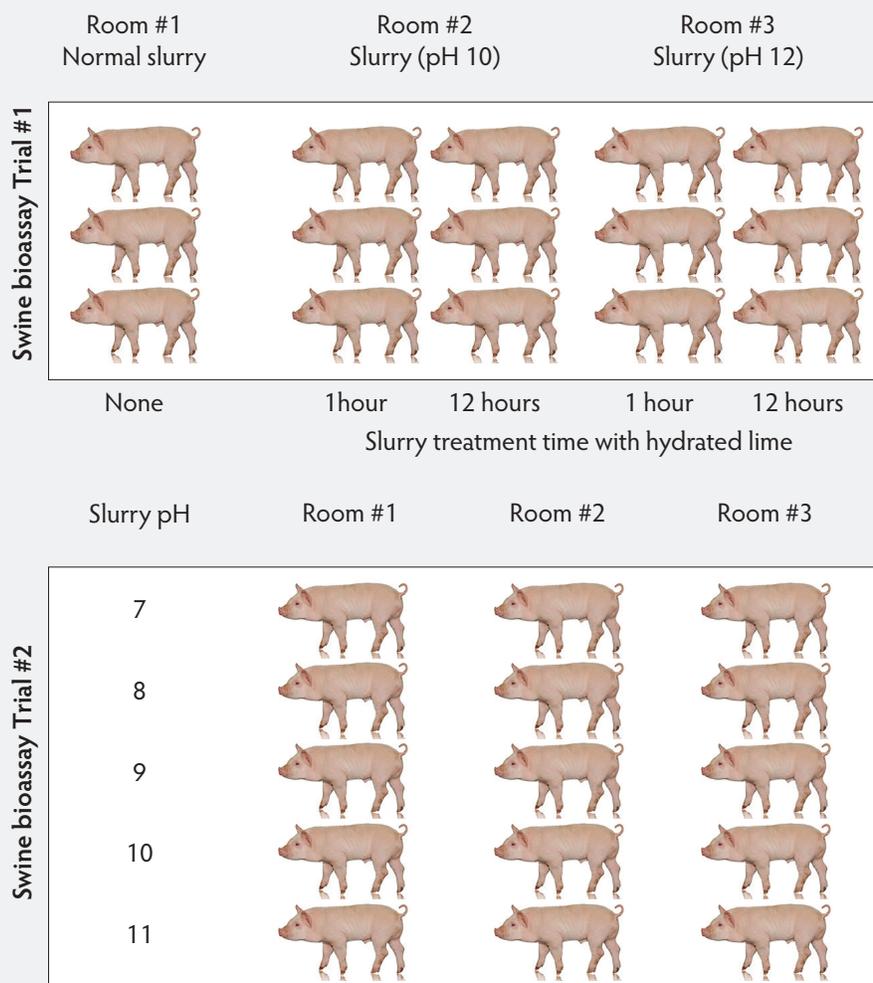
Table 1: Effect of hydrated lime manure treatment exposure (1 or 12 hours) at pH 10 or 12 on porcine epidemic diarrhea virus (PEDV) abundance and potential to cause disease

| Treatment	Time (hours)	Slurry PEDV* log/gram	Pig bioassay†	
			IHC (%)	Rectal swab (Cq)
None	0	9.16 ± 0.02^a	3/3 (100)	20.4, 23.4, 20.7
pH 10	1	7.00 ± 0.36^b	0/3 (0)	All > 40
	12	5.38 ± 0.33^c	0/3 (0)	All > 40
pH 12	1	4.5 ± 0.01^c	0/3 (0)	All > 40
	12	BD	0/3 (0)	All > 40

* Log RNA targets/g wet manure slurry determined by reverse transcriptase quantitative PCR $\pm$ 1 SE; (Cq > 40 $\approx$ 10^4 per gram in manure slurry or 25 copies/PCR reaction).

† IHC was performed as previously described.[9] Cq = quantification cycle for rectal swab at necropsy. Cq values $\leq$ 35 were considered positive and > 35 were considered negative.

abc Values within a column with different superscripts are significantly different ($P < .05$; ANOVA).

BD = below detection; IHC = immunohistochemistry.

Figure 2: Effect of increasing hydrated lime amendment during alkaline stabilization on swine manure slurry pH and PEDV genome abundance assessed using reverse transcriptase quantitative PCR. Error bars = 1 SE; PEDV = porcine epidemic diarrhea virus; PCR = polymerase chain reaction.
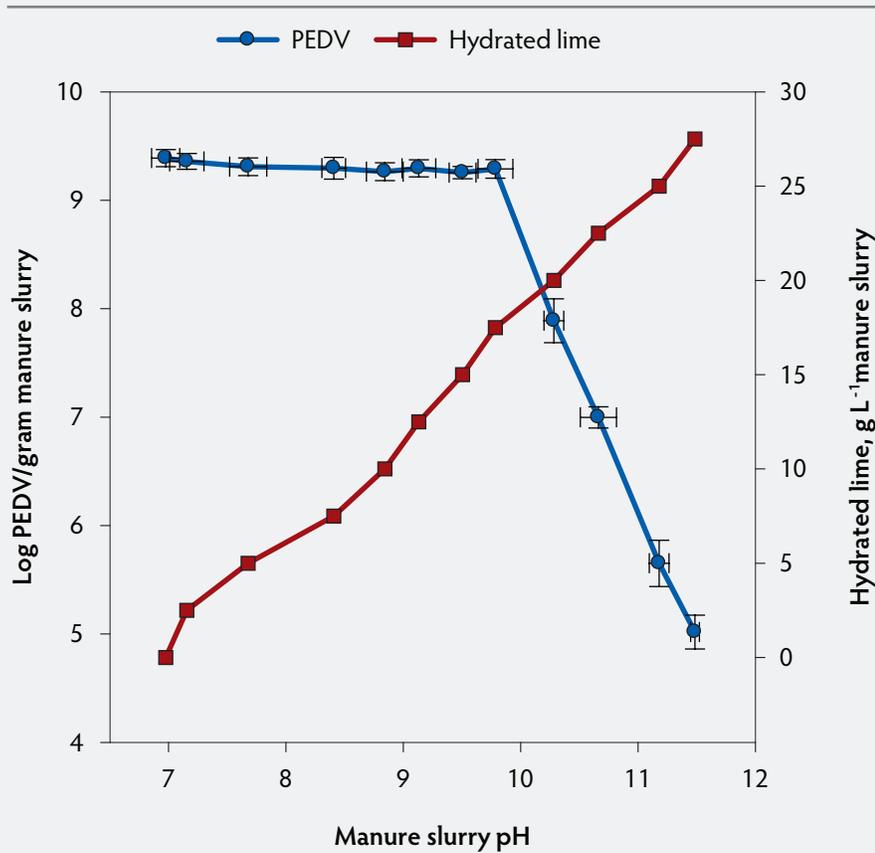
Discussion

Alkaline stabilization was achieved in manure initially containing 10^9 PEDV targets per gram of slurry at and above pH 10 (ie, infectivity was eliminated). Comparing the pig bioassay results with RT-qPCR results, an interesting relationship emerges. Although reduced by more than 100-fold above pH 10, PEDV target genomes could still be detected at 10^5 to 10^7 per gram of slurry. Alkaline stabilization impeded virus infection but did not destroy all past evidence of the presence of the virus (ie, some remnant RNA persisted for a short period of time). Alkaline pH likely altered virus envelope integrity, which released PEDV RNA into the manure slurry where RNA was quickly hydrolyzed. Not all animals exposed to PEDV-contaminated manure treated below pH 10 became infected with PEDV, particularly animals in the second study. It was noted that the pigs in the second manure slurry trial were slightly larger than those in the first trial, and this may account for the lower incidence of disease in pigs exposed to manure slurry below pH 10.

Ammonium increased by a substantial fraction in the third manure slurry incubation, particularly in the lower pH treatments (8, 8.5, and 9). Decomposition processes in the lower pH fresh manure (urea hydrolysis and organic matter decomposition) likely account for the increase, while higher pH may have inhibited these decomposition processes. Additionally, in the manure samples of higher pH (9.5 and 10), the dissociation of ammonium to ammonia (pK_a 9.25) would shift ammonium to ammonia, which is more

Table 2: Effect of incremental hydrated lime addition on manure slurry pH and PEDV abundance and potential to cause disease

Hydrated lime (g/L)	Average pH	Slurry PEDV* log/g	Pig bioassay†	
			IHC (%)	Rectal swab (Cq)
0.0	6.92	9.36[a]	1/3 (33)	> 40; 25.93; > 40
6.7	8.14	9.26[a]	1/3 (33)	25.49; > 40; > 40
12.5	9.13	9.23[a]	2/3 (67)	23.25; >40; 23.31
18.3	9.96	8.66[a]	0/3 (0)	All > 40
24.2	11.07	6.15[b]	0/3 (0)	All > 40

*   Log RNA targets/g wet manure slurry determined by reverse transcriptase quantitative PCR.
†   IHC performed as previously described.[9] Cq = quantification cycle for rectal swab at necropsy. Cq values ≤ 35 were considered positive and > 35 were considered negative.
[ab] Values within a column with different superscripts are significantly different ($P < .05$, ANOVA).
PEDV = porcine epidemic diarrhea virus; IHC = immunohistochemistry.

easily lost via volatilization. Although simulated storage (PIT versus TANK) showed no difference, slurry pH had a dramatic effect on ammonium concentrations (up to 25% difference between low and high pH) after a short incubation period.

While "lime" is a term broadly used to describe calcium-containing inorganic materials, "quicklime" applies to the chemical compound calcium oxide (CaO), which is unstable and highly reactive to moisture. To reduce the reactivity of quicklime and make it more stable, water is often added to quicklime to convert all oxides of calcium and magnesium to hydroxides. The resulting compound, calcium hydroxide [$Ca(OH)_2$], is sold under a number of different names, including hydrated lime, slaked lime, caustic lime, and others. Among other applications, hydrated lime is commonly used during the cleanup phase after a disease outbreak in livestock production systems. Despite being more stable than quicklime, hydrated lime is still caustic and quick to react with water, so it must be handled with care. Precautions should be taken to protect against inhalation or contact with skin and eyes. In addition to keeping arms and legs covered, gloves, safety goggles and a dust mask should be worn during handling.

To accomplish alkaline treatment of manure slurry at a swine production site using hydrated lime, a dosing rate of approximately 23 kg (50 lb) of hydrated lime per 3800 L (1000 gal) of manure is recommended. At a cost of around $40 for a 50-lb bag, treatment of a full 5000-gal slurry tank spreader can be accomplished for approximately $200 (USD). Addition of the hydrated lime to the tank wagon prior to it being filled with slurry is recommended to facilitate mixing. Addition of lime while a slurry tank is being filled with manure is not recommended, since the concentration of ammonia gas emanating from the tank wagon fill port could be high enough to cause asphyxiation for the person adding lime at the tank port. While the research presented included an analysis of ammonia loss during treatment of stored slurry, the addition of hydrated lime to deep pit manure storages is not recommended. The substantial amount of ammonia gas generated during alkalization of an entire manure pit containing several thousand gallons of manure slurry may pose a significant health risk to workers and animals in and near the production facility.

Implications

- Alkaline stabilization through hydrated lime addition to achieve a threshold pH 10 for 1 hour is sufficient to deactivate the porcine epidemic diarrhea virus in manure slurry on the basis of bioassay outcomes. Although PEDV was still detectable above pH 10 by RT-qPCR (10^5 to 10^7 genomes per gram manure slurry), no disease risk was observed.
- Important questions remain regarding the minimum treatment time needed for alkaline stabilization and whether longer treatment periods at < 10 pH are as efficacious as briefer, higher pH treatment.
- Raising manure slurry pH above 9.25 will likely enhance ammonia losses by volatilization and decrease fertilizer N value. Alkaline stabilization of manure slurry could present a risk for ammonia asphyxiation during manure treatment and pumping if proper air flow is inadequate.

Conflict of interest

JDL, co-author, has served as a consultant for, and thus has disclosed a significant financial interest in, Harrisvaccines, Inc. In accordance with its Conflict of Interest policy, the University of Nebraska-Lincoln's Conflict of Interest in Research Committee has determined that this must be disclosed.

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CONVERSION TABLES

Weights and measures conversions

Common (US)	Metric	To convert	Multiply by
1 oz	28.35 g	oz to g	28.4
1 lb (16 oz)	453.59 g	lb to kg	0.45
2.2 lb	1 kg	kg to lb	2.2
1 in	2.54 cm	in to cm	2.54
0.39 in	1 cm	cm to in	0.39
1 ft (12 in)	0.31 m	ft to m	0.3
3.28 ft	1 m	m to ft	3.28
1 mi	1.6 km	mi to km	1.6
0.62 mi	1 km	km to mi	0.62
1 in²	6.45 cm²	in² to cm²	6.45
0.16 in²	1 cm²	cm² to in²	0.16
1 ft²	0.09 m²	ft² to m²	0.09
10.76 ft²	1 m²	m² to ft²	10.8
1 ft³	0.03 m³	ft³ to m³	0.03
35.3 ft³	1 m³	m³ to ft³	35
1 gal (128 fl oz)	3.8 L	gal to L	3.8
0.264 gal	1 L	L to gal	0.26
1 qt (32 fl oz)	946.36 mL	qt to L	0.95
33.815 fl oz	1 L	L to qt	1.1

Temperature equivalents (approx)

°F	°C
32	0
50	10
60	15.5
61	16
65	18.3
70	21.1
75	23.8
80	26.6
82	28
85	29.4
90	32.2
102	38.8
103	39.4
104	40.0
105	40.5
106	41.1
212	100

$$°F = (°C × 9/5) + 32$$
$$°C = (°F - 32) × 5/9$$

Conversion chart, kg to lb (approx)

Pig size	Lb	Kg
Birth	3.3-4.4	1.5-2.0
Weaning	7.7	3.5
	11	5
	22	10
Nursery	33	15
	44	20
	55	25
	66	30
Grower	99	45
	110	50
	132	60
Finisher	198	90
	220	100
	231	105
	242	110
	253	115
Sow	300	135
	661	300
Boar	794	360
	800	363

1 tonne = 1000 kg
1 ppm = 0.0001% = 1 mg/kg = 1 g/tonne
1 ppm = 1 mg/L