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A feasibility study of conducting surveillance for swine pathogens in slurry from North Carolina swine farms

Emily S. Bailey[1,2,3]✉, Laura K. Borkenhagen[1,2], Jessica Y. Choi[1,2], Annette E. Greer[4], Marie R. Culhane[5] & Gregory C. Gray[1,2,6,7]

Despite close contact between humans and animals on large scale farms, little to no infectious disease research is conducted at this interface. Our goal in this preliminary study was to explore if we could detect swine pathogens using a non-invasive, indirect approach through the study of swine slurry. From April to November 2018, 105 swine slurry samples were collected by farm personnel from waste pits at two sites on a swine farm in North Carolina. These samples were tested for DNA and RNA viruses using a real-time PCR and RT-PCR. Statistical analyses were performed to measure association between virus positive outcomes and potential predictors such as date of sample collection, weight of pigs, number of pigs in barn, temperature, and weather conditions. Overall, 86% of the samples had evidence of at least one of the targeted viruses. Ultimately, this study demonstrated the utility of conducting noninvasive surveillance for swine pathogens through the study of swine slurry. Such swine slurry surveillance may supplant the need to handle, restrain, and collect specimens directly from pigs thus providing an approach to emerging pathogen detection that appeals to the swine industry.

In farm environments, humans and animals are in frequent close contact where they are known to exchange zoonotic pathogens[1]. Despite this knowledge, sparse pathogen surveillance is conducted at this human–animal interface. Instead, emerging zoonotic pathogens are usually only detected when a pathogen's impact is severe in either the animals or the animal workers that clinical investigations are subsequently sought. This results in missed opportunities for early detection and mitigation efforts.

Finding ways to collaborate with animal production industries is key to conducting such human-animal interface surveillance. Major industry objections that must be overcome include the biosecurity risks of permitting researchers to enter farms and the harm that the specimen collection may cause the animals. As there is increasing evidence that zoonotic viruses may be transmitted via environmental pathways such as through aerosol, feces, and water[2–7], in this preliminary study we sought to engage swine farmers in periodically collecting fecal slurry samples from swine farms and to evaluate those samples for molecular evidence of zoonotic swine pathogens[8–10]. In this study the zoonotic pathogens are those that have the potential to pass between swine and humans, including influenza viruses, enteroviruses, coronaviruses, adenoviruses, and encephalomyocarditis virus. Such indirect and noninvasive fecal slurry surveillance reduces both the threats of biosecurity breaches and potential harm rendered in sampling production animals. These methods provided collection consistency and proved that established trustworthy partnerships of research engagement hold hope for future expansion of One Health research models. Our overall goal was to determine if our slurry sampling might be an acceptable

[1]Duke Global Health Institute, Duke University, Durham, NC, USA. [2]Division of Infectious Diseases, Duke University School of Medicine, Durham, NC, USA. [3]Julia Jones Matthews Department of Public Health, Graduate School of Biomedical Sciences, Texas Tech University Health Sciences Center, 1650 Pine Street, Abilene, TX 79601, USA. [4]Brody School of Medicine Department of Bioethics and Interdisciplinary Studies, East Carolina University, North Carolina Agromedicine Institute, Greenville, NC, USA. [5]Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St. Paul, MN, USA. [6]Global Health Research Center, Duke-Kunshan University, Kunshan, China. [7]Emerging Infectious Diseases Program, Duke-NUS Medical School, Singapore, Singapore. ✉email: emily.bailey@ttuhsc.edu

method of conducting pathogen surveillance at the human-animal interface and yield robust calculations of prevalence of detected pathogens.

Results

From the months of April through November 2018, a total of 105 swine slurry samples were collected from two sites on a farm in Eastern North Carolina. Overall, 90 (86%) of the 105 total swine slurry samples had evidence of at least one zoonotic virus. Four samples were positive for porcine reproductive and respiratory syndrome virus (PPRSv) (4%), one was positive for encephalomyocarditis virus (EMCV) (1%), three were positive for porcine circovirus 2 (PCV2) and PCV3 (3% respectively), and 20 samples (19%) were positive for senecavirus. 48 (46%) slurry samples were positive for adenovirus, 62 (59%) were positive for enterovirus, 39 (37%) samples were positive for coronavirus, and no samples were positive for influenza A, B, C, or D. 45 (94%) of 48 adenovirus positive specimens were successfully sequenced using partial genome sequencing and found to have clonal evidence of porcine adenovirus 5 (NCBI accession number AF289262.1). 42 (68%) of the 62 enterovirus positive specimens were successfully sequenced and were found to be a variety of human and porcine enteroviruses (Table 1), including porcine enteroviruses, human coxsackieviruses and echoviruses.

Enterovirus was the most prevalent virus with the greatest number of detections made during the months August through November. There were also multiple detections of adenovirus, coronavirus, and senecavirus. There were few detections of PRRSv, PCV2, PCV3, and EMCV. Bivariate risk factor studies were conducted for adenovirus, enterovirus, coronavirus, senecavirus, and any viral detection (Table 2). Adenovirus positivity was sometimes related to lower temperature and coronavirus positivity was related to extreme weather. Detections of both enterovirus and coronavirus were found to be associated with pig weight and the number of pigs in the barn, with more observed positives among the lowest three weight quartiles (45–295 lbs). Detections of adenovirus were found to be associated with the lowest weight quartile. Finally, detection of coronavirus and senecavirus often coincided with detection of enterovirus (OR 3.8; 95% CI 1.6, 9.2 and OR 3.6; 95% CI 1.1, 11.5, respectively).

Discussion

In this preliminary study, we conducted molecular surveillance for viruses in swine slurry samples collected from farms in Eastern North Carolina. Our overall goal was to determine if non-invasive slurry sampling was useful in the farm setting in detecting swine pathogens and supported by the farm owners. As such, the farmer was able to collect, freeze, and ship samples to our laboratory in Durham, North Carolina. Our unique approach of engaging farmers in developing research questions and including them in the presentation of results is representative of a shift in research focusing on animal production. This type of approach, better termed the One Health approach, brings together researchers across disciplines for the improvement of human, animal, and environmental health. The research presented here demonstrated that farmers are interested and willing to participate in research, so much so, that the farmer was willing to collect additional data on weather, number of pigs in the barn, etc. Although research working with farmers should consider specific factors such as farmer training, biosecurity, sample processing, and result dissemination, the relationships that come from working together to determine what questions are important to individuals growing food production animals are valuable for both parties and for public health as a whole.

Through our surveillance, we were able to identify the presence of multiple zoonotic viruses in slurry samples, such as coronaviruses and enteroviruses. Although, it is likely that influenza A virus was circulating within the swine population on the farm[3], we were not able to identify this virus in the waste samples. Anderson et al., 2018 identified low concentrations of influenza A in swine slurry samples from farms in China; however, efforts to culture these viruses were not successful[3]. Similar to the results of our study, the largest detections of influenza in the Anderson et al., 2018 study were detected when the numbers of pigs on the farm were the greatest. In contrast to our results, Anderson et al., 2018 found that weather and temperature patterns were significantly related to positive detections of influenza A.

We did find considerable molecular evidence of adenovirus, enterovirus, and coronavirus using our pan-species molecular detection methods. These results are similar to other studies that have examined the species diversity of microorganisms in both swine and human waste[11]. Of particular interest is the detection of both animal and human enteroviruses (Table 1). We posit that these viruses were either present in water or food products given to the pigs or that there somehow was viral transmission between swine workers and the swine herd[12].

Our analysis of temperature and weather indicates that in general these predictors were associated with increased adenovirus and coronavirus detections but that swine weight and the number of pigs in the barn were suggestive of increased enterovirus and coronavirus viral RNA positivity.

This research was limited by the lack of virus isolation data. As this was a preliminary study, virus isolation was not our goal. Samples have been archived for further characterization including infectivity experiments. Additionally, the statistical analysis was limited by the lack of a full year of data, which may have revealed additional viral associations with the seasonality of weather and temperature. Another limitation of this study is the lack of comparison to standard techniques used for swine pathogen sampling, a logical next step would be to perform a more complete comparison of this method to more traditional oral secretion detection methods.

Overall our findings demonstrated that zoonotic viruses (enteroviruses and coronaviruses) can be readily detected in swine slurry samples; as such, these samples may be used as an alternative non-invasive method for virus surveillance on swine farms. Future research directions should include a paired sample approach to link viral swine infections (through samples of swine nasal or oral secretions or feces) with swine waste.

Sample ID	Date collected	Enterovirus type	Accession number from NCBI GenBank
2	4/24/2018	Porcine enterovirus B	GQ502354.1
4	4/26/2018	Porcine enterovirus B	AM261011.1
5	5/1/2018	Enterovirus A NIE2014	KT717068.1
6	5/1/2018	Enterovirus G	KT265893.2
11	5/9/2018	Enterovirus G	KT265893.2
12	5/9/2018	Enterovirus G	KF705669.1
20	5/24/2018	Porcine enterovirus B	GQ502354.1
45	7/18/2018	Enterovirus G	MF113342.1
48	7/25/2018	Porcine enterovirus B	AM261011.1
49	7/25/2018	Coxsackievirus A4	KX021215.1
53	8/1/2018	Porcine enterovirus B	AM261020.1
61	8/14/2018	Enterovirus G	KF705660.1
62	8/16/2018	Enterovirus G	KY761948.1
66	8/23/2018	Porcine enterovirus B	GQ502354.1
69	8/30/2018	Porcine enterovirus B	AM261011.1
70	9/4/2018	Enterovirus A NIE2014	KT717068.1
71	9/4/2018	Enterovirus G	KT265893.2
73	8/27/2018	Enterovirus G	KT265893.2
76	9/16/2018	Enterovirus G	KT265946.1
77	9/16/2018	Human echovirus 11	JQ654098.1
78	9/18/2018	Enterovirus G	KT265973.1
79	9/18/2018	Echovirus E27	KC787137.1
80	9/6/2018	Echovirus JAA-2013	KC787146.2
83	9/25/2018	Enterovirus G	LC316821.1
85	9/27/2018	Enterovirus G	KY761948.1
86	10/2/2018	Coxsackievirus A10	KP164191.1
87	10/2/2018	Porcine enterovirus 10	JX219532.1
88	10/3/2018	Coxsackievirus A10	KP164191.1
89	10/3/2018	Enterovirus G	KT265882.1
91	10/9/2018	Porcine enterovirus 10	JX219532.1
92	10/11/2018	Coxsackievirus A10	KP164191.1
93	10/11/2018	Enterovirus G	KT265893.2
95	10/16/2018	Enterovirus G	KT265910.1
96	10/18/2018	Enterovirus G	KT265961.2
97	10/18/2018	Coxsackievirus B1	KU560979.1
99	10/27/2018	Enterovirus G	KT265961.2
100	11/1/2018	Enterovirus G	KP982873.1
101	11/1/2018	Porcine enterovirus 10	JX219532.1
102	11/7/2018	Enterovirus G	KP982873.1
103	11/7/2018	Porcine enterovirus 10	JX219532.1
104	11/8/2018	Coxsackievirus A10	KP164191.1

Table 1. Molecular subtyping results for enterovirus positive swine slurry samples.

Materials and methods

Site enrollment. A North Carolina, USA, swine farm with two barns was identified to participate in this preliminary study. Each barn had 12 pens with a center hallway and a fully slatted concrete floor over a deep pit to hold feces, urine, and waste water. Pits were emptied up to three times per year and recharged with recycled water. Farm personnel collected up to two slurry samples per week from the pit. A survey was completed for each sampling session and included information regarding date of collection, sampling site, sampling time, weight of pigs at site, number of pigs, and weather condition.

Sample collection and processing. Slurry is defined as the feces and urine from pigs and the waste water used to remove the urine and feces from the pig pens[13]. Slurry samples from two swine barns containing finishing pigs were collected from approximately 5–10 cm below the surface of pits and were frozen at − 20 °C until shipped to our laboratory (a maximum of 24 h). Frozen samples and completed surveys were transported

Predictor	Adenovirus		Enterovirus		Coronavirus		Senecavirus		Any positives[a]	
	No. (%)	OR (95% CI)	No. (%)	OR (95% CI)	No. (%)	OR (95% CI)	No. (%)	OR (95% CI)	No. (%)	OR (95% CI)
Month										
July	1 (8.3)	0.10 (0.01, 1.02)	5 (41.7)	4.64 (0.71, 30.42)	3 (25.0)	1.33 (0.22, 8.22)	0 (0.0)	–	8 (66.7)	1.00 (0.20, 5.00)
August	6 (30.0)	0.50 (0.12, 1.97)	10 (50.0)	**6.50 (1.16, 36.57)**	6 (30.0)	1.71 (0.35, 8.37)	4 (20.0)	0.46 (0.11, 1.94)	8 (66.7)	2.83 (0.55, 14.47)
September	6 (42.9)	0.86 (0.20, 3.71)	13 (92.9)	**84.50 (6.80, 1,050.80)**	5 (35.7)	2.22 (0.42, 11.83)	9 (64.3)	3.34 (0.80, 13.94)	17 (85.0)	–
October	8 (57.1)	1.52 (0.35, 6.60)	13 (92.9)	**84.50 (6.80, 1,050.80)**	13 (92.9)	52.00 (0.474, 570.53)	0 (0.0)	–	14 (100.0)	–
November	3 (50.0)	1.14 (0.17, 7.60)	5 (83.3)	**32.50 (2.38, 443.14)**	6 (100.0)	–	0 (0.0)	–	6 (100.0)	–
April	4 (100.0)	–	4 (100.0)	–	0 (0.0)	–	0 (0.0)	–	4 (100.0)	–
May	12 (60.0)	1.71 (0.44, 6.63)	9 (45.0)	5.32 (0.94, 29.99)	3 (15.0)	0.71 (0.12, 4.11)	7 (35.0)	Ref	15 (75.0)	1.50 (0.34, 5.56)
June	7 (46.7)	Ref	2 (13.3)	Ref	3 (20.0)	Ref	0 (0.0)	–	10 (66.7)	Ref
Weather										
Sun	29 (44.6)	1.29 (0.38, 4.36)	38 (58.5)	1.64 (0.50, 5.43)	24 (36.9)	3.22 (0.66, 15.77)	9 (13.8)	0.88 (0.17, 4.66)	53 (81.5)	1.32 (0.32, 5.56)
Sun & wind	3 (37.5)	0.96 (0.16, 5.90)	5 (62.5)	1.94 (0.32, 11.76)	6 (75.0)	**16.50 (1.83, 148.61)**	4 (50.0)	5.50 (0.71, 42.60)	7 (87.5)	2.10 (0.18, 24.60)
Cloudy/overcast	8 (66.7)	3.20 (0.62, 16.49)	6 (50.0)	1.17 (0.24, 5.62)	5 (41.7)	3.93 (0.59, 26.11)	2 (16.7)	1.10 (0.13, 9.34)	12 (100.0)	–
Rain & wind	2 (28.6)	0.64 (0.09, 4.66)	6 (85.7)	1.94 (0.32, 11.71)	2 (28.6)	2.20 (0.24, 20.40)	3 (42.9)	4.12 (0.49, 34.49)	6 (85.7)	1.80 (0.15, 21.40)
Rain	5 (38.5)	Ref	6 (46.2)	Ref	2 (15.4)	Ref	2 (15.4)	Ref	10 (76.9)	Ref
Temperature (°F)										
< 70	6 (75.0)	**8.00 (1.25, 51.14)**	6 (75.0)	3.60 (0.59, 21.93)	4 (50.0)	2.14 (0.41, 11.17)	1 (12.5)	0.49 (0.05, 4.94)	8 (100.0)	–
70–79	4 (33.3)	1.33 (0.29, 6.12)	10 (83.3)	6.00 (1.06, 34.00)	7 (58.3)	3.00 (0.70, 12.88)	2 (16.7)	0.68 (0.11, 4.18)	10 (83.3)	1.11 (0.17, 7.17)
80–89	31 (49.2)	2.58 (0.89, 7.46)	35 (55.6)	1.50 (0.57, 3.98)	21 (33.3)	1.07 (0.38, 3.03)	12 (19.0)	0.80 (0.25, 2.60)	52 (82.5)	1.05 (0.30, 3.72)
90 +	6 (27.3)	Ref	10 (45.4)	Ref	7 (31.8)	Ref	5 (22.7)	Ref	18 (81.8)	Ref
Pigs weight (lbs)										
45–95 (Q1)	5 (16.1)	**0.18 (0.05, 0.61)**	14 (45.2)	2.12 (0.69, 6.51)	9 (29.0)	3.00 (0.72, 12.59)	6 (19.4)	1.76 (0.39, 7.89)	25 (80.6)	2.34 (0.70, 7.85)
100–200 (Q2)	14 (53.8)	1.08 (0.36, 3.24)	21 (80.8)	**10.80 (2.92, 39.99)**	12 (46.2)	**6.29 (1.50, 26.31)**	8 (30.8)	3.26 (0.75, 14.12)	25 (96.2)	**14.06 (1.62, 121.84)**
220–295 (Q3)	15 (65.2)	1.73 (0.54, 5.54)	19 (82.6)	**12.21 (3.05, 48.91)**	15 (65.2)	**13.75 (3.13, 60.42)**	3 (13.0)	1.10 (0.20, 6.09)	22 (95.6)	**12.38 (1.42, 107.74)**
300 (Q4)	13 (52.0)	Ref	7 (28.0)	Ref	3 (12.0)	Ref	3 (12.0)	Ref	16 (64.0)	Ref
Number of pigs										
1,000 +	22 (53.7)	1.81 (0.82, 3.99)	32 (78.0)	**4.29 (1.77, 10.43)**	16 (39.0)	1.14 (0.51, 2.56)	9 (22.0)	1.36 (0.51, 3.63)	35 (85.4)	1.21 (0.41, 3.57)
< 1,000	25 (39.1)	Ref	29 (45.3)	Ref	23 (35.9)	Ref	11 (17.2)	Ref	53 (82.8)	Ref
Adenovirus										
Positive	–	–	32 (68.1)	2.13 (0.96, 4.75)	18 (38.3)	1.09 (0.49, 2.42)	9 (19.2)	1.01 (0.38, 2.69)	–	–
Negative	–	–	29 (50.0)	Ref	21 (36.2)	Ref	11 (19.0)	Ref	–	–
Enterovirus										
Positive	32 (52.5)	2.13 (0.96, 4.75)	–	–	30 (49.2)	**3.76 (1.55, 9.15)**	16 (26.2)	**3.56 (1.10, 11.52)**	–	–
Negative	15 (34.1)	Ref	–	–	9 (20.4)	Ref	4 (9.1)	Ref	–	–
Coronavirus										
Positive	18 (46.2)	1.09 (0.49, 2.42)	30 (76.9)	**3.76 (1.55, 9.15)**	–	–	6 (15.4)	0.68 (0.24, 1.93)	–	–
Negative	29 (43.9)	Ref	31 (47.0)	Ref	–	–	14 (21.2)	Ref	–	–
Senecavirus										
Positive	9 (45.0)	1.01 (0.38, 2.69)	16 (80.0)	**3.56 (1.10, 11.52)**	6 (30.0)	0.68 (0.23, 1.93)	–	–	–	–
Negative	38 (44.7)	Ref	45 (52.9)	Ref	33 (38.8)	Ref	–	–	–	–

Table 2. Unadjusted odds ratios for risk factors associated with virus positivity among 105 swine slurry samples. Bold text represents significant results. Samples were collected from swine waste pits at two pig farms in North Carolina between April and November 2018. *No.* number of positives, *OR* unadjusted odds ratio, *CI* confidence interval, *Ref.* referent group, *Q* quartile. [a]Any positives outcome denotes one or more virus (adenovirus, enterovirus, coronavirus, encephalomyocarditis virus, porcine circovirus 2, porcine circovirus 3, porcine reproductive and respiratory syndrome virus, and senecavirus) detected in sample.

overnight to the Duke One Health Research Laboratory. Dates and pre-assigned sample numbers were used for sample tracking.

Slurry samples were diluted by methods previously described[14–16]. Briefly, samples were diluted at 10% w/v in sterile phosphate buffered saline (PBS) (pH 7.2). All samples were centrifuged for 1 min at 1,000*g* and 5 mL of the supernatant was centrifuged at 4 °C for 30 min at 1,500*g*. The remaining supernatant (~ 1.5 mL) was transferred

to a sterile Eppendorf tube and centrifuged at 12,000*g* for 10 min. Finally, 1 mL of the supernatant was stored at − 80 °C until molecular testing was performed.

Laboratory testing. We adapted previously published techniques for molecular evidence of both DNA and RNA viruses. Viral DNA was extracted from slurry samples using the QIAamp DNA Blood Mini Kit (QIAGEN, Inc., Valencia, CA) and tested with a real-time PCR (qPCR) assay for porcine circovirus 2 (PCV2)[17] and porcine circovirus 3 (PCV3)[18] using SsoAdvanced Universal Probes Supermix Real-Time PCR kit (BioRad, Inc., Hercules, CA). Viral DNA was also assessed using gel-based PCR assays with the Platinum Taq DNA Polymerase Kit (Thermo Fisher Scientific, Inc., Waltham, MA) for the detection of pan-species adenovirus[19].

Viral RNA was extracted from slurry samples using the QIAamp Viral RNA Mini Kit (QIAGEN, Inc., Valencia, CA), and then assessed with real-time RT-PCR (qRT-PCR) assays using the SuperScript III Platinum One-Step qRT-PCR System with Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Inc., Waltham, MA) for the detection of influenza A[20], influenza B[21], influenza C[22], influenza D[23], and encephalomyocarditis virus (EMCV)[24]. For the detection of porcine reproductive and respiratory syndrome virus (PRRSv), Tetracore EZ-PRRSv rRT-PCR assay was used (Tetracore, Inc., Rockville, MD). Additionally, viral RNA was assessed with gel-based RT-PCR assays using the SuperScript III Platinum One-Step RT-PCR System with Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Inc., Waltham, MA) for the detection of pan-species enterovirus[25], pan-species coronavirus (unpublished), and senecavirus[26]. Cell culture was not attempted for these specimens. Partial genome sequencing was performed by Eton Bioscience (Eton Bioscience, Inc., Raleigh, NC, USA) for positive specimens. Sequences were then compared to the NCBI sequence database using the BLAST application of BioEdit 7.1.9 (Ibis Biosciences, Carlsband, CA, USA). Sequences were aligned and phylogenetic analysis was performed using the UPGMA method in Geneious Prime 2019.1.1 (Biomatters Inc., San Diego, CA, USA).

Statistical analysis. Chi-square test and Fisher's exact test were performed to measure statistical association between potential predictors of the outcome of molecular assay positivity for each individual virus, as well as molecular evidence for any one or multiple viruses. Potential predictors included farm number, month of sample collection, time of sample collection, weight of pigs, number of pigs in barn, temperature, and weather conditions, as well as positivity for other viruses. Odds ratios and 95% confidence intervals were calculated for potential risk factors. Statistical analyses were performed using STATA 15.0 (StataCorp, College Station, TX).

Ethics approval. This study was granted exemption from review status by the IACUC at Duke University on the grounds that the research did not include direct sample collection from animals.

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Author contributions

E.B. and A.G. conducted sampling collection. E.B. and J.C. processed samples. L.B. supported data analysis. G.G. and M.C. reviewed and edited the manuscript. All authors have reviewed and approved the final text.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to E.S.B.

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Laboratory-scale inactivation of African swine fever virus and swine vesicular disease virus in pig slurry

C. Turner[1] and S.M. Williams[2]

[1]*Silsoe Research Institute, Bedford, and* [2]*Institute for Animal Health, Pirbright, UK*

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C. TURNER AND S.M. WILLIAMS. 1999. Two methods were evaluated for the inactivation of African swine fever (ASV) and swine vesicular disease (SVD) viruses in pig slurry: chemical treatment and heat treatment. The addition of NaOH or $Ca(OH)_2$ at different concentration/time combinations at 4 °C and 22 °C was examined, as was virus stability at different temperature/time combinations. ASF virus (ASFV) was less resistant to both methods than SVD virus (SVDV). In slurry from one source, ASFV was inactivated at 65 °C within 1 min, whereas SVDV required at least 2 min at 65 °C. However, it was found that thermal inactivation depended on the characteristics of the slurry used. Addition of 1% (w/v) of NaOH or $Ca(OH)_2$ caused the inactivation of ASFV within 150 s at 4 °C; 0·5% (w/v) NaOH or $Ca(OH)_2$ required 30 min for inactivation. NaOH or $Ca(OH)_2$ (1% (w/v)) was not effective against SVDV at 22 °C after 30 min, and 1·5% (w/v) NaOH or $Ca(OH)_2$ caused inactivation of SVDV at both 4 °C and 22 °C. At higher chemical concentrations or temperatures, ASFV and SVDV inactivation was faster in slurry than in buffered medium.

INTRODUCTION

African swine fever (ASF) is a highly contagious viral disease of wild and domestic pigs. In endemic areas (the southern half of Africa), wild pigs may show no symptoms of the disease. However, in the domestic pig, the situation is quite different. Here, ASF can be very serious, with some strains of the virus causing 100% mortality, although since spreading from Africa to Europe, the virulence of some isolates of ASF has decreased, with a consequent reduction in mortality rates. This has led to virus being carried by some apparently healthy recovered animals (Wilkinson 1981) and these may pose a risk to healthy pig populations. ASF virus (ASFV) was present in southern Europe from its arrival in Portugal from Africa in 1957, until it was eradicated from Portugal in 1993 and from Spain in 1995. It is still present in Sardinia.

ASFV is a large, enveloped, icosahedral DNA virus of approximately 200 nm in size. It is generally quite resistant to inactivation, and can survive many cycles of freezing and thawing. It is resistant to pH changes, and a proportion of the population of some isolates can survive at pH 4 and 13 (Plowright and Parker 1967). It is, however, very sensitive to drying, and is readily inactivated by lipid solvents because of its envelope.

The first known outbreak of swine vesicular disease (SVD) was in Italy in 1966 (Nardelli *et al*. 1968), although it may have had another origin. Since then, there have been many outbreaks across Europe, with Great Britain and Italy being particularly affected (Hedger and Mann 1989). SVD, while not usually a fatal disease, is highly contagious and produces clinical signs that are indistinguishable from foot and mouth disease (mild fever and vesicles on coronary bands of foot and skin of limbs), and therefore is considered a serious problem for differential diagnosis of the two diseases. SVD virus (SVDV) is a member of the Picornaviridae, being small (about 30 nm), icosahedral and lacking an envelope. It is relatively stable over a wide pH range (from pH 2–12), can survive many days without loss of infectious virus titre and is resistant to many forms of inactivation.

In the UK, in the interests of safeguarding the national herd, government policy is that outbreaks of either disease require the slaughter of pig herds and the decontamination of buildings and anything that has come into contact with the animals, including pig slurry. The trend towards increasingly large and intensively reared pig herds means that many farms have insufficient land for the immediate land disposal of pig slurry and manure. This, coupled with the fact that winter

Correspondence to: Dr C. Turner, Silsoe Research Institute, Wrest Park, Silsoe, Bedford MK45 4HS, UK (e-mail: claire.turner@bbsrc.ac.uk).

slurry spreading carries additional pollution risks, means that pig slurry is frequently stored for 4–6 months prior to land disposal. The Ministry of Agriculture, Fisheries and Food in the UK (MAFF 1991) strongly recommend a storage time of at least 4 months. As a result, many pig farms have large slurry stores (often up to 5000 tonnes), and should a disease outbreak occur, the slurry stores are likely to be contaminated by the disease agent. Hence, an outbreak of ASF or SVD is likely to require the decontamination of large quantities of animal slurry and/or solid manure. Slurry provided the initial focus for the investigation of suitable decontamination techniques, and this study was confined to investigations into the inactivation of virus in slurry.

There are many ways of inactivating viruses. These include physical methods, such as the application of heat (Herniman *et al*. 1973; Monteith *et al*. 1986) or ionizing radiation (Vasl *et al*. 1983; Farooq *et al*. 1993), chemical methods using chlorine (Lothrop and Sproul 1969; Bosch *et al*. 1993), ozone (Warriner *et al*. 1985), acids, alkalis etc. (Herniman *et al*. 1973), biological methods such as the action of bacteria or proteases (Deng and Cliver 1995), or the use of aerobic (Munch *et al*. 1987) or anaerobic treatment (Monteith *et al*. 1986). Other techniques involve physically removing the virus from the liquid medium, for instance, using sand column filtration (Powelson and Gerba 1994). Although nearly all of these methods are suitable for use in water or aqueous solutions with low dry matter (DM) content, only a limited number may be suitable for use with large quantities of a liquid containing substantial levels of dry matter, such as animal (pig) slurry. Others, such as ozonation or u.v. irradiation, could have benefit if the slurry is pre-clarified. The use of gamma irradiation is limited to certain viruses (SVDV is considered resistant) unless very high doses are used, and the human health risks at large scale would be considerable. The same concern applies to ozonation and the use of many chemicals, including formalin, which is highly toxic.

Considering the factors of efficacy and reliability plus the relative costs and ease of scale-up and slurry disposal after treatment, two techniques were identified as being potentially suitable for the inactivation of ASFV and SVDV in pig slurry: heat treatment, and dosing with an alkaline chemical, specifically sodium hydroxide or calcium hydroxide (Turner and Burton 1997). These methods are relatively easy to scale up, generally inexpensive, and the treated slurry (especially after heat treatment) can be disposed of in the usual way, i.e. by land spreading.

Experimental objectives and design

The objectives of this investigation were to evaluate two alternative methods of virus inactivation: heat treatment and the addition of calcium or sodium hydroxide. Both approaches were applied to pig slurries inoculated with ASFV or SVDV, to determine the best method for virus inactivation, and to identify the necessary levels of treatment to meet a prescribed standard of inactivation under practical conditions. The level of inactivation required in these experiments was set at a 10^4-fold reduction of infectious virus titre. This inactivation level was set because it is the standard which disinfectants have to meet in order to be certified for use against specific viruses in the UK. It would have been impossible to verify a treatment's efficacy if inoculating with low levels that would be likely to occur in a field situation (due partly to the relatively high minimum detectable levels), so a high titre inoculum was necessary to reveal the effectiveness of the inactivation process.

Heat treatment and experimental design. Virus was incubated in slurry at various temperatures for different times to: (i) determine how stable ASFV and SVDV were at different temperatures over several hours in both slurry and EMEM; (ii) determine at which temperatures ASFV and SVDV are inactivated in slurry within 15 min; and (iii) determine the virus inactivation profile of ASFV and SVDV in slurry at different temperatures over 2–5 min.

Slurry from two different sources was used in the heating experiments. Experiments with slurry from one of the sources (source 2) were performed in triplicate and in this case, slurry samples were diluted by tap water to achieve the different concentrations of total solids.

Chemical addition and experimental design. The approach here was to find a range of chemical concentrations for each virus which would lead to inactivation within 30 min, and then to determine the concentrations that would act rapidly, to cause inactivation within 150 s. All chemical inactivation experiments were performed using slurry from a single source (source 1).

MATERIALS AND METHODS

Virus isolates and stock preparation

ASFV isolate from Malawi, designated Lilongwe 20/1 (Haresnape 1984), was obtained from infected pig spleen, which was macerated with sterile sand and added to 90 ml Eagle's minimal essential medium (EMEM) containing 1% ox serum and 1% penicillin/streptomycin (antibiotic) solution (containing penicillin at 10 000 U ml^{-1} and streptomycin at 10 $\mu g\,ml^{-1}$). The mixture was clarified by centrifugation to remove gross particles and stored at 4 °C. The SVDV strain used was a tissue culture adapted strain (UKG25/72) grown in IB-RS2 (renal swine) cells.

Assays

Given the number of samples generated for virus assay, a detection system allowing multiple assays was required. For the assay of ASFV, a microtitre plate system was chosen, using 96 well plates seeded with 100 μl of $1{\cdot}5 \times 10^{7{\cdot}0}$ ml^{-1} pig bone marrow (PBM) cells in Earle's saline solution. A plaque assay in IB-RS2 (pig kidney) cells was used to detect SVDV, using confluent cell sheets on 6 well plates.

ASFV assay. Samples were serially diluted 10-fold in virus diluent (phosphate-buffered saline (PBS) containing 1% antibiotic solution, 1% ox serum and 0·1% phenol red). Four replicates of each dilution (50 μl well^{-1}) were added to PBM cell cultures. The plates were sealed, shaken gently and incubated for 6 d at 37 °C in air containing 5% CO_2. At the end of this incubation period, the wells were examined microscopically for haemadsorption (HAD) and any well containing haemadsorbing cells was designated positive. To improve the sensitivity of the assay, 20 μl of a fresh 1% erythrocyte suspension was then added and the plates were incubated for a further 3 d prior to final examination. The infectious virus titre (HAD_{50} ml^{-1}) was calculated from the number of positive wells observed at each dilution. In EMEM, the limit of detection was $10^{1{\cdot}3}$ HAD_{50} ml^{-1}, which corresponds to two out of four positive wells at zero dilution. Titres below this where haemadsorption occurred (one positive well at zero dilution) were described as 'trace'.

SVDV assay. Samples were serially diluted 10-fold in virus diluent, and 200 μl of each dilution were added, in duplicate, to a confluent monolayer of IB-RS2 cells on each well of a 6 well plate. These cells were incubated in an incubator providing 5% CO_2 in air at 37 °C for 1 h and were then overlaid with 2 ml Eagle's overlay medium supplemented with 2% serum, 1% antibiotics solution and 0·8% Noble agar. After incubating for a further 48 h, the infectious virus titre (pfu ml^{-1}) was determined following staining with 2 ml methylene blue solution, counting plaques and adjusting to get an infectious virus titre ml^{-1}. In EMEM, the limit of detection was $10^{0{\cdot}7}$ pfu ml^{-1}.

Inoculation of slurry with virus. Slurry was seeded with virus to give a 10% (v/v) virus suspension and mixed gently.

Recovery of virus from slurry. ASFV: Equal volumes of ox serum and slurry containing ASFV were combined, stirred for 30 min, centrifuged at 7000 *g* for 1 min and the supernatant fluid assayed as described above. The lowest detectable level for virus extracted in this way was $10^{1{\cdot}8}$ HAD_{50} ml^{-1}. *SVDV*: Equal volumes of Freon (1,1,2 trichlorofluoroethane) and slurry–virus suspension were combined, vigorously shaken for a few minutes, and centrifuged at 8000 *g* for 90 s. The top layer was then assayed as described above. The lowest detectable level for virus extracted and assayed in this way was $10^{0{\cdot}7}$ pfu ml^{-1}.

Slurries

Two sources of slurry were used in these experiments from two commercially operated pig farms, and these were designated Source 1 and Source 2. Slurry from Source 1 had the following average characteristics (the assays according to APHA (1985)). Total solids (TS) content: 2·3%; chemical oxygen demand (COD): 36 gl^{-1}; Kjeldahl nitrogen concentration: 2·8 gl^{-1}; ammoniacal nitrogen concentration: 2·1 gl^{-1}. Slurry from Source 2 had the following average characteristics (prior to dilution). TS content: 5·0%; COD: 60 gl^{-1}; Kjeldahl nitrogen concentration: 2·7 gl^{-1}; ammoniacal nitrogen concentration: 1·8 gl^{-1}. Slurry from Source 2 was diluted with tap water in experiments (performed in triplicate) to determine the effect of dilution on virus inactivation and was used at the following TS concentrations: 5%, 2·5%, 1% and 0·5%. Uninfected slurry was assayed prior to use for the presence of viruses that could interfere with assays, and found to be negative.

Heating experiments

Slurry or EMEM was pre-heated at the required temperature in 25 ml glass bottles placed in a water bath prior to inoculation with virus. When the temperature had equilibrated, virus was added to the slurry or EMEM at a time designated time zero (t = 0), and the required temperature was maintained. Samples were removed at the required time intervals, extracted, and assayed according to the procedure described above.

Chemical addition experiments

Granular NaOH and powdered $Ca(OH)_2$ used in these experiments were obtained from BDH Chemicals. Each sample used to assess the effect of chemical concentration was prepared by adding slurry from Source 1 to an appropriately weighed amount of either chemical in a 25 ml glass bottle. Each experiment was initiated when virus suspension was added to achieve a concentration of 10% v/v. The duration of each experiment was measured from the moment of virus addition (designated t = 0). The pH of the slurry was measured before and after chemical addition, and immediately following the required duration of chemical treatment, the pH of the suspension was restored to its starting value by adding 5 mol l^{-1} HCl to prevent death of the cell sheets used in the virus assays. Control treatments were performed by

adding the chemical, then slurry or EMEM, then restoring the starting pH before addition of virus to determine whether the 5 mol l^{-1} HCl addition had any effect on the virus.

The experiment to determine the efficacy of NaOH or $Ca(OH)_2$ against ASFV in EMEM used the following concentrations of chemical at 4 °C or 22 °C: NaOH at 1%, 0·5%, 0·2% and 0·1% (w/v) and $Ca(OH)_2$ at 2%, 1%, 0·5% and 0·2% (w/v). In slurry with ASFV, $Ca(OH)_2$ was used at 1% and 0·5% (w/v); NaOH was used at 1%, 0·5% and 0·2% (w/v) at 4 °C and 22 °C. In both these experiments, the chemical was allowed to act for a period of 30 min before neutralization. The inactivation of ASFV over 150 and 300 s was also examined, where NaOH or $Ca(OH)_2$ was added to ASFV in slurry containing 0·5% (w/v) of either chemical at 22 °C, and 1% (w/v) of either chemical used at 4 °C with samples being taken after 150 and 300 s before neutralization.

For SVDV inactivation in EMEM, NaOH and $Ca(OH)_2$ were added at concentrations of 0·2%, 0·5% and 1% (w/v) to vials containing EMEM, mixed, and neutralized after 30 min. A similar experiment was performed with NaOH and $Ca(OH)_2$ at 1%, 0·5% and 0·2% (w/v) in slurry for 30 min. The experiment to determine the effect of NaOH and $Ca(OH)_2$ on SVDV in slurry over a short time period was performed as follows: slurry containing 1% and 1·5% (w/v) of either chemical was inoculated with 10% (v/v) SVDV at 22 °C, and samples taken at 150 s and 300 s and immediately neutralized with 5 mol l^{-1} HCl.

RESULTS

Heat inactivation

ASFV. (i) The stability of ASFV in EMEM at 4, 22, 40, 50 and 60 °C over 24 h was examined (Fig. 1). It can be seen that the virus was relatively stable at 4, 22 and 40 °C, losing less than 10^1 HAD_{50} ml^{-1}. At 50 °C, however, the virus titre declined steadily, so that after 5 h, the titre was $10^{1·8}$ HAD_{50} ml^{-1} and after 24 h, only a trace of virus remained. At 60 °C, no virus could be detected 15 min after inoculation. In slurry from Source 1, the virus titre declined more rapidly, and the titre was below detectable levels after only 4 h at 40 °C and within 1 h at 50 °C (Fig. 2). Again, no virus could be detected after 15 min at 60 °C.

(ii) ASFV was incubated in slurry from Source 1 at 50, 53, 56 and 60 °C. Virus was only detected at 50 and 53 °C up to 15 min, and after 30 min, no virus was detected at any of the temperatures. These results demonstrated that virus inactivation in slurry occurs at a more rapid rate than it does in EMEM.

(iii) Having ascertained that the temperatures at which ASFV in slurry from Source 1 was inactivated to below detectable levels within 15 min was between 53 and 60 °C, ASFV was incubated in slurry from Source 1 at 56 and 60 °C to determine the lowest temperature at which ASFV is inactivated within 90 s, and it was found that ASFV was inactivated within 30 s at 60 °C, and within 90 s at 56 °C. In an experiment with slurry from Source 2 at different TS concentrations, ASFV was incubated at 56, 60 and 65 °C for up to 5 min. In this experiment, it was found that ASFV was not inactivated as readily as in the slurry from Source 1 (Table 1). At 56 °C, inactivation to below detectable levels occurred after 2–5 min. At 60 °C, inactivation occurred between 2 and 3 min for 5, 2·5 and 1% TS; at 0·5% TS, it occurred between 3 and 4 min. At 65 °C, ASFV was inactivated at all TS concentrations within 60 s. From these results, it seems that the source of slurry (and hence its constituents) affected the speed of ASFV inactivation at different temperatures. There also appeared to be a marginally

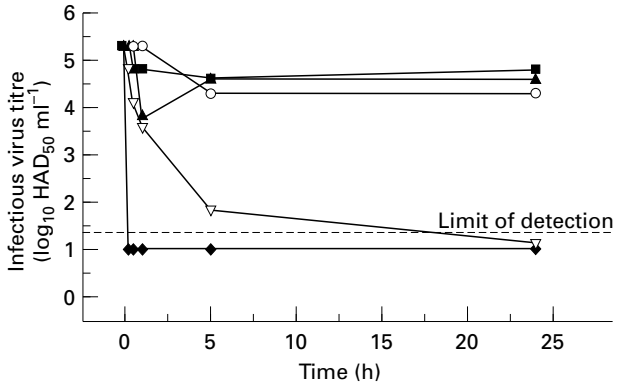

Fig. 1 Thermal inactivation of ASFV in EMEM after 24 h. Initial virus titre: $10^{5·3}$ HAD_{50} ml^{-1}. Titres that could not be detected are shown at just below the limit of detection. A trace of virus was detected after 24 h at 50 °C. (■), 4 °C; (○), 22 °C; (△), 40 °C; (▽), 50 °C; (◆), 60 °C

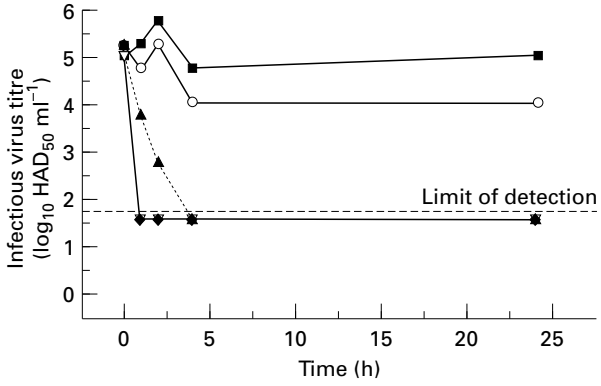

Fig. 2 Thermal inactivation of ASFV in pig slurry after 24 h. Initial virus titre: $10^{5·3}$ HAD_{50} ml^{-1}. Titres that could not be detected are shown at just below the limit of detection. (■), 4 °C; (○), 22 °C; (▲), 40 °C; (▽), 50 °C; (◆), 60 °C

Table 1 Inactivation of ASFV in slurry from Source 2 at 5%, 2·5%, 1% and 0·5% TS at 56, 60 and 65 °C

%TS	Sample time (min)	56 °C	60 °C	65 °C
0·5%	0	5·4 (5·1–5·6)	5·6 (5·3–6·3)	4·5 (4·3–4·8)
	0·5	5·5 (5·3–5·8)	NT	⩽1·8 (⩽1·8–⩽1·8)
	1	5·4 (5·1–5·8)	5·6 (4·6–6·3)	⩽1·8 (⩽1·8–⩽1·8)
	2	5·2 (5·1–5·3)	5·0 (4·6–5·8)	NT
	3	NT	<3·3 (⩽1·8–3·3)	NT
	4	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	5	⩽1.8 (⩽1.8–trace)	⩽1·8 (⩽1·8–⩽1·8)	NT
1%	0	5·3 (5·1–5·6)	5·1 (4·8–5·3)	4·6 (4·3–4·8)
	0·5	5·3 (5·1–5·6)	NT	<4·6 (⩽1·8–4·6)
	1	5·4 (5·1–5·6)	4·0 (3·1–5·1)	⩽1·8 (⩽1·8–⩽1·8)
	2	5·2 (5·1–5·3)	<2·3 (⩽1·8–2·3)	NT
	3	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	4	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	5	⩽1·8 (⩽1·8–⩽1·8)	⩽1·8 (⩽1·8–⩽1·8)	NT
2·5%	0	5·7 (5·3–6·1)	5·3 (5·3–5·3)	3·6 (2·6–4·6)
	0·5	5·3 (5·1–5·6)	NT	<2·8 (⩽1·8–2·8)
	1	5·4 (5·3–5·6)	4·3 (2·8–5·3)	⩽1·8 (⩽1·8–⩽1·8)
	2	5·2 (4·8–5·6)	<3·6 (⩽1·8–3·6)	NT
	3	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	4	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	5	<2·1 (⩽1·8–2·1)	⩽1·8 (⩽1·8–⩽1·8)	NT
5%	0	5·4 (5·1–5·6)	5·4 (5·3–5·6)	4·3 (⩽1·8–4·3)
	0·5	5·4 (5·1–5·6)	NT	⩽1·8 (⩽1·8–⩽1·8)
	1	5·2 (4·8–5·6)	3·6 (2·8–4·1)	⩽1·8 (⩽1·8–⩽1·8)
	2	5·2 (5·1–5·3)	⩽1·8 (⩽1·8–⩽1·8)	NT
	3	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	4	NT	⩽1·8 (⩽1·8–⩽1·8)	NT
	5	⩽1·8 (⩽1·8–⩽1·8)	⩽1·8 (⩽1·8–⩽1·8)	NT

Three replicates were tested and values given are the mean (and range).
Virus titres are given as $\log_{10}$ HAD_{50} ml^{-1}. Virus added had a titre of $10^{6·8}$ HAD_{50} ml^{-1}, and was added at a 10-fold dilution.
NT = not tested.

greater stability of ASFV in slurry at 0·5% TS compared with 1, 2·5 and 5% TS.

SVDV. (i) The stability of SVDV over 24 h was examined in both EMEM and slurry from Source 1 at the following temperatures: 4, 22, 40, 50 and 60 °C. In EMEM, results showed that SVDV was stable over 24 h at 4, 22 and 40 °C. However, the titre started to fall after 1 h at 50 °C and was not detectable after 4 h (Fig. 3). In slurry from Source 1, SVDV was stable at 4 and 22 °C; at 40 °C, the titre had started to decline over 24 h. No virus was detectable in slurry incubated at 50 or 60 °C (Fig. 4), where (apart from at t = 0) the first sample was taken after 1 h.

(ii) In a separate experiment to find the temperature at which SVDV was inactivated within 15 min, SVDV incubated in slurry from Source 1 survived for up to 1 h at 50 °C, although the decline from a start titre of 10^7 pfu ml^{-1} was rapid. At 56 °C, SVDV survived in slurry from Source 1 for less than 15 min.

(iii) Where SVDV was incubated in slurry from Source 1 at both 56 and 60 °C, it was found that SVDV at 56 °C at a start titre of $10^{7·95}$ pfu ml^{-1} survived for at least 5 min (although the titre had declined to $10^{1·5}$ pfu ml^{-1}). At 60 °C, a trace of SVDV was detectable after 90 s, although not after 2 min. These experiments indicated that SVDV in slurry from Source 1 is inactivated to below detectable levels within 2 min at 60 °C. In slurry from Source 2, where slurry samples (in triplicate) at different TS concentrations were heated to

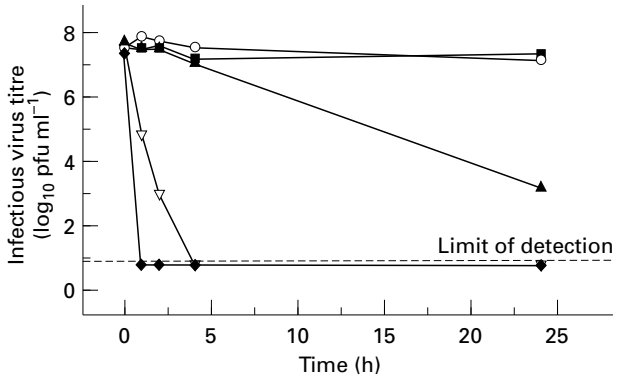

Fig. 3 Thermal inactivation of SVDV in EMEM after 24 h. Initial virus titre: $10^{7\cdot7}$ pfu ml^{-1}. Titres that could not be detected are shown at just below the limit of detection. (■), 4 °C; (○), 22 °C; (▲), 40 °C; (▽), 50 °C; (◆), 60 °C

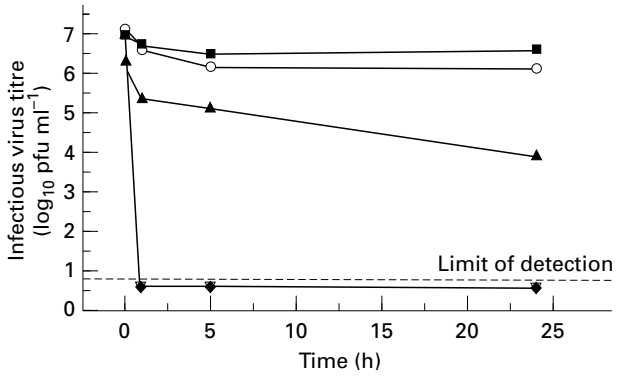

Fig. 4 Thermal inactivation of SVDV in pig slurry after 24 h. Initial virus titre: 10^{7} pfu ml^{-1}. Titres that could not be detected are shown at just below the limit of detection. (■), 4 °C; (○), 22 °C; (▲), 40 °C; (▽), 50 °C; (◆), 60 °C

60 and 65 °C, it was found that slurry was inactivated within 5 min at 60 °C at all slurry dilutions except 0·5% TS, and within 2 min at 65 °C except in one replicate at 5% TS. Results are given in Table 2.

These experiments indicated that as with ASFV, SVDV is more heat labile in slurry than in EMEM. They also demonstrated that the thermal inactivation of viruses is strongly dependent on the characteristics of the slurry.

Chemical inactivation

ASFV. Addition of NaOH and $Ca(OH)_2$ to virus suspensions in EMEM at 4 and 22 °C showed that $Ca(OH)_2$ was effective at both temperatures at a concentration of 1% (w/v); NaOH was effective at 0·5% (w/v), although a trace of virus was present after 30 min in 1% NaOH at 4 °C. Temperature had an effect on the chemical inactivation of ASFV, with

Table 2 Inactivation of SVDV in slurry from Source 2 at 5%, 2·5%, 1% and 0·5% TS at 60 and 65 °C

%TS	Sample time (min)	60 °C	65 °C
0·5%	0	6·8 (6·4–7·2)	4·8 (4·4–5·0)
	1	5·6 (5·5–5·9)	<0·7 (<0·7–<0.7)
	2	3·6 (3·0–4·5)	<0·7 (<0·7–<0.7)
	3	2·3 (2·0–2·7)	NT
	4	2·4 (1·4–3·2)	NT
	5	<2·0 (<0·7–2·4)	NT
1%	0	6·0 (5·6–6·3)	4·1 (4·1–4·2)
	1	5·1 (4·5–5·5)	<1·4 (<0·7–1·4)
	2	4·4 (2·9–5·4)	<0·7 (<0·7–<0.7)
	3	2·4 (1·7–3·7)	NT
	4	2·7 (1·7–3·3)	NT
	5	<0·7 (<0·7–<0.7)	NT
2·5%	0	6·6 (6·2–6·8)	3·3 (3·2–3·6)
	1	5·2 (5·0–5·4)	<0·7 (<0·7–<0.7)
	2	2·8 (2·7–3·0)	<0·7 (<0·7–<0.7)
	3	<0·7 (<0·7–<0.7)	NT
	4	<0·7 (<0·7–<0.7)	NT
	5	<0·7 (<0·7–<0.7)	NT
5%	0	7·2 (7·2–7·3)	5·7 (5·6–6·0)
	1	6·6 (6·6–6·7)	1·8 (1·4–2·4)
	2	6·0 (5·9–6·1)	2·1 (<0·7–2·1)
	3	5·0 (4·3–5·4)	NT
	4	4·3 (4·2–4·3)	NT
	5	<0·7 (<0·7–<0.7)	NT

Three replicates were tested and values given are the mean (and range).
Virus titres are given as $\log_{10}$ pfu ml^{-1}. Virus added had a titre of $10^{9\cdot0}$ pfu ml^{-1} at a 10-fold dilution.
NT = not tested.

inactivation occurring at lower concentrations of chemical at 22 than at 4 °C (Fig. 5). In slurry, it was found that ASFV was inactivated by $Ca(OH)_2$ within 30 min at 1% and 0·5% (w/v) at 4 and 22 °C; NaOH was effective at 1, 0·5 and 0·2% (w/v) at 22 °C, and at 1 and 0·5% (w/v) at 4 °C, 0·2% being ineffective at this temperature.

As with the heat inactivation studies, following an initial evaluation of concentration ranges likely to be effective, it was necessary to find a concentration of chemical that could act rapidly. A study of the inactivation of ASFV in slurry (of initial titre $10^{5\cdot8}$ HAD_{50} ml^{-1}) by NaOH or $Ca(OH)_2$ after 150 and 300 s demonstrated that 1% (w/v) of either chemical at 4 °C rapidly inactivated ASFV to below detectable levels, but 0·5% (w/v) at 22 °C was relatively ineffective after 5 min

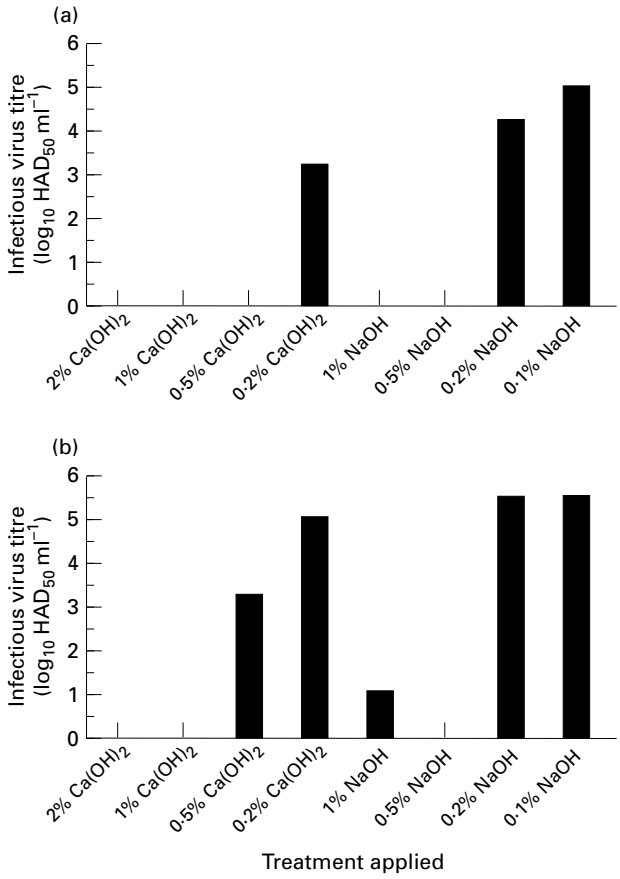

Fig. 5 Inactivation of ASFV in EMEM by $Ca(OH)_2$ and NaOH at (a) 22 °C and (b) 4 °C

(Table 3). These results indicated that virus inactivation occurred at lower concentrations of NaOH or $Ca(OH)_2$ in slurry than in EMEM. This is in spite of the fact that slurry buffers the system much better than does EMEM, and addition of either alkali led to smaller pH changes in slurry. For example, the addition of 1% (w/v) NaOH to EMEM changed its pH from 7·4 to 12·9, while the same concentration in slurry increased the pH from 7·8 to 10·6. This implies that the action of these chemicals was not a pH effect alone.

SVDV. NaOH and $Ca(OH)_2$ at concentrations effective for the inactivation of ASFV (0·2, 0·5 and 1% (w/v)) were used against SVDV in EMEM 22 °C with the initial SVDV titre being $10^{7.1}$ pfu ml^{-1}. NaOH and $Ca(OH)_2$ were less effective at these concentrations against SVDV than against ASFV (Fig. 6). The effect of NaOH and $Ca(OH)_2$ against SVDV in slurry at 22 °C over 30 min was examined and results show that 1% (w/v) of NaOH and $Ca(OH)_2$ was effective, but lower concentrations did not produce sufficient virus inactivation (Table 4).

Having identified chemical concentrations that would cause inactivation of SVDV over 30 min, as with ASFV, it was necessary to find a concentration of chemical that would

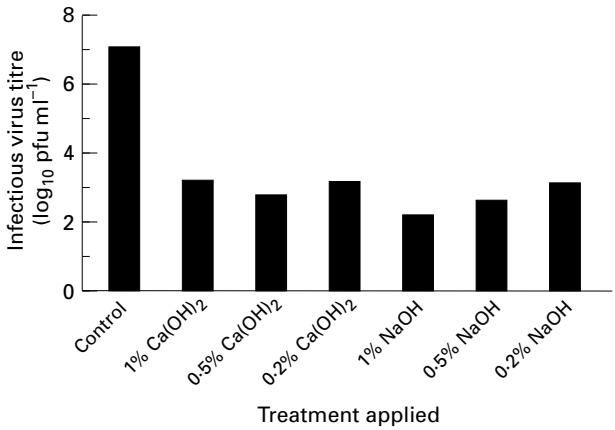

Fig. 6 Inactivation of SVDV in EMEM by $Ca(OH)_2$ and NaOH at 22 °C and 4 °C. Control virus titre shown at $10^{7.1}$ pfu ml^{-1}

Table 3 The inactivation of ASFV in slurry by chemicals over 5 min

Chemical and temperature	Treatment time (s)	Virus titre ($\log_{10}$ HAD_{50} ml^{-1})
0·5% NaOH, 22 °C	150	4·1
	300	3·6
0·5% $Ca(OH)_2$, 22 °C	150	3·1
	300	3·3
1% NaOH, 4 °C	150	$\leqslant$1·8
	300	$\leqslant$1·8
1% $Ca(OH)_2$, 4 °C	150	$\leqslant$1·8
	300	$\leqslant$1·8

Start titre = $10^{6.7}$ pfu ml^{-1}

Table 4 Inactivation of SVDV after 30 min in slurry with chemicals

Chemical treatment	Final concentration (% w/v)	Virus titre ($\log_{10}$ pfu ml^{-1})
None-control	0	7·2
$Ca(OH)_2$	1	$<0·7$
	0·5	7·0
	0·2	6·1
NaOH	1	0·7
	0·5	6·3
	0·2	7·1

act rapidly, i.e. within 150 s. Hence the effect of NaOH and $Ca(OH)_2$ at a concentration of 1% (w/v) on SVDV in slurry over a short time period (150 and 300 s) was studied. After 300 s, virus still persisted in the slurry, although the titre was reduced (Table 5). At 1·5% (w/v), either chemical at 4 and 22 °C inactivated SVDV to below detectable levels within 150 s.

In a control experiment in which slurry samples containing 1·5% (w/v) NaOH were neutralized with 5 mol l^{-1} HCl prior to SVDV addition, the HCl had no adverse affect on the virus, indicating that it was not responsible for virus inactivation. However, some loss of virus titre ($10^{1·5}$ pfu ml^{-1}) occurred when slurry samples containing $Ca(OH)_2$ were neutralized with HCl.

DISCUSSION

The results presented here demonstrated that either chemical inactivation or heat treatment can inactivate both ASFV and SVDV in pig slurry and EMEM. SVDV was more resistant to both treatments than ASFV, requiring a greater concentration of chemicals or higher temperature to achieve similar inactivation. SVDV was inactivated to below detectable levels by 1·5% (w/v) of either NaOH or $Ca(OH)_2$ whereas ASFV required only 1% (w/v) of either chemical to achieve similar inactivation. With heat treatment, generally higher temperatures or longer time periods were required for the inactivation of SVDV compared to ASFV.

The time taken for treatment to be effective is an important consideration in devising a method for large-scale inactivation of virus in slurry. If the treatment method is to be applied to the decontamination of a large volume of slurry, the faster the decontamination can be achieved, the quicker and easier the treatment of the entire volume of slurry will proceed. A given treatment method should also render the material safe within a quantifiable safety margin. In other words, it should not only be possible to demonstrate the effectiveness of a particular treatment, but also to determine the minimum level of treatment to achieve a particular reduction in virus titre. Apart from identifying suitable safety margins, this approach will also prevent a process from being 'over-engineered' at greater expense.

An interesting feature of the results is that slurry, or components of it, appears to enhance the effects of the virus inactivation treatment. ASFV, for instance, was inactivated to below detectable levels within 1 h at 50 °C in slurry from Source 1, whereas similar inactivation in EMEM requires more than 24 h. ASFV levels remained stable over 24 h at 40 °C in EMEM but in slurry from Source 1, the titre declined to below detectable levels within 4 h. The pattern was similar for SVDV. These results imply that the inactivation demonstrated was not due simply to the effect of heat alone, but may have been assisted by the release of

Table 5 Stability of SVDV in slurry at chemical concentrations of 1% at 22 °C after 150 and 300 s

Chemical treatment	Duration of treatment (s)	Virus titre ($\log_{10}$ pfu ml^{-1}
1% NaOH	150	2·5
	300	2·1
1% $Ca(OH)_2$	150	2·8
	300	3·0

Start titre = $10^{6·7}$ pfu ml^{-1}

virucidal agents, possibly ammonia, in the slurry when it was heated. The mechanism of temperature inactivation of ASFV and SVDV appeared to be different. When exposure to high temperature was limited to 90 s, ASFV was inactivated at a lower temperature than SVDV. However, ASFV appeared to survive longer at sub-lethal temperatures (e.g. 50 °C) than SVDV. This can be seen by comparing Figs 1 and 2 with Figs 3 and 4.

Thermal virus inactivation also appeared to be dependent on the nature or source of the slurry. Results obtained with both ASFV and SVDV showed that the source of the slurry can have an affect on the inactivation time. ASFV and SVDV were both inactivated more rapidly and at a lower temperature in slurry from Source 1 than in slurry from Source 2. The reason for this was not further investigated, and conclusions could not easily be drawn from the differences in quoted characteristics of the slurries, such as chemical oxygen demand, total solids and nitrogen content. However, in these and in subsequent experiments (data not shown), it was shown that both ASFV and SVDV were consistently inactivated at higher temperatures in cell growth medium or water than in slurry. Therefore, in order to be confident that virus inactivation will occur, it is recommended that the treatment temperature is slightly higher than that shown to be effective in any of the media tested.

Results from chemical inactivation were also not entirely predictable. It was believed that the inactivation occurred as a result of an increase of pH to above 12, which was the case when NaOH or $Ca(OH)_2$ was used at concentrations above 0·5% (w/v) in EMEM. However, the slurry had a strong pH buffering effect, and the pH only rose to 10·6 when 1% (w/v) NaOH was added, compared with a pH of 12·9 when added to EMEM, and yet higher concentrations of chemicals were required for ASFV inactivation in slurry than in medium. Therefore, it appeared that virus inactivation by NaOH or $Ca(OH)_2$ was not merely a pH effect.

Although the use of NaOH or $Ca(OH)_2$ and the application of heat are all capable of inactivating ASFV and SVDV to below detectable levels in pig slurry, chemical treatment is likely to be a less suitable method for use with large volumes of slurry due to the difficulties in ensuring that all parts of the slurry are adequately mixed and come into contact with the required concentration of chemicals for the duration of treatment. Disposal of chemical-contaminated slurry is also a potential problem when large volumes are involved. For small volumes, however, either method is adequate for ASFV or SVDV inactivation.

Hence, based on the results of this research, the recommendation is that large quantities of contaminated pig slurry are heat treated at a temperature of 65 °C for a minimum period of 5 min. This treatment will ensure that a reasonable margin of safety is applied to the treatment process to account for differences in the characteristics of the slurry which have been shown to have an effect on virus inactivation. This would be an expensive proposition if batch processing was used. However, with continuous flow processing, engineering techniques can be used to recover much of the heat required.

The differences observed in inactivation profiles in different media, and even in similar but not identical media (e.g. slurry from two sources) under different conditions demonstrate that the process of inactivation is more complex than a mere temperature or pH effect. Hence, a complete picture of relevant inactivation data needs to be taken into account when designing a large-scale inactivation process for a particular virus.

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Spreading manure: Understanding the potential disease impact

Casey Zangaro, Erica Rogers, Michigan State University Extension - July 15, 2019

> **Help prevent the spreading of diseases by putting proper manure biosecurity practices in place**

It is important to consider biosecurity practices when moving manure on and off the farm

With a wet spring delaying both manure application and planting, many farmers are working hard to catch up on field work. As farmers work to put manure on their fields, proper biosecurity practices should be taken into consideration.

Why is manure biosecurity important?

Biosecurity is important for the prevention of disease transmission and protection of animal health. Most swine operations have in-depth biosecurity protocols in place to safeguard the health of their herd such as, downtime prior to entering the facility and isolation periods for incoming stock. Another important consideration when building your biosecurity plan is understanding how diseases may spread through manure application. Viruses such as Porcine Epidemic Diarrhea Virus (PEDv) and African Swine Fever Virus (ASF) have been shown to be transmittable in swine manure. The awareness of how every aspect of daily farm operations can affect biosecurity is becoming ever more important.

Producers need to consider the movement of potentially infectious manure across farmland and popular farm road routes. The movement of manure has the capacity to move diseases between farms or sites within an operation, as roadways are shared by various farm operations.

What manure hauling biosecurity practices could be implemented?

The following are best management biosecurity practices for manure handling:

- Institute proper cleaning and disinfection protocols for manure equipment; this means proper cleaning/washing to remove organic material before applying an appropriate sanitizer or disinfectant.
  - Clean, then disinfect manure equipment inside and out when moving between manure sources, different livestock sites, or fields.
  - If there is a concern of disease spread from infectious manure, have tire washing and disinfecting stations established at all possible points of entry.
- Have dedicated routes of travel when moving manure and communicate with those using similar routes for their farm's manure application.
  - If spreading infected manure, avoid roadways commonly used by other livestock operations.
  - Plan ahead and have an open dialogue with neighboring livestock farms regarding disease status.

        - For example, if spreading infected manure, plan to use fields away from main roadways and livestock areas.
    - Properly maintain equipment so that manure spills are prevented as you travel to and from your destination.
        - Should a spill occur, contact the Agriculture Pollution/Spills Hotline at 1-800-405-0101.
    - Utilize best management practices during land application of manure that help prevent field run off as outlined in the Michigan Right to Farm Manure Management and Utilization Generally Accepted Agricultural Management Practices (GAAMPs):
        - Incorporate manure within 48 hours of application if possible.
        - Avoid excessive application rates (apply for crop nutrient needs – soil and manure analysis).
        - Avoid frozen or snow covered ground.
        - Utilize conservation practices for prevent erosion losses to surface waters.
        - Be aware of slopes within a field (the larger the slope, the more likely the occurrence of runoff).
        - Avoid land application to saturated soils.
        - Avoid land application right before and during heavy times of precipitation.
        - Utilize tools to help determine runoff risk like the Michigan EnviroImpact Tool.
    - Have dedicated equipment, applicator clothing and footwear when completing manure transfer and application.
    - Wear different clothing and boots between sites or utilize disposable coveralls and booties where applicable.
    - Create designated footpaths and vehicle routes on the premises that diminish crossover between employees/visitors entering the facilities and those responsible for manure application and transport.
    - Understand that downtimes and health pyramids between farm sites also apply to equipment and transport vehicles.
        - Typically farms request 48-72 hours of time between visits to different sites or a completed cleaning and disinfection process of the manure transport equipment.
        - Custom applicators should visit with their contracted operations to make sure that they are following the proper site entry protocols.
    - Make sure each farm has a Standard Operating Procedure for Biosecurity and follow accordingly.
    - Keep complete records of dates of manure agitation, removal, locations visited and fields where manure was applied.
        - When working with different farm operations, a list of all sites, species housed there and dates of application/visits should be made available upon request for

the producers you are working with. This is important information for tracking disease spread and biosecurity breaches.

The Future: research-based strategies to reduce virus loads in manure

Animal slurry contains antibacterial and antiviral properties (ex. ammonia) that can change the pH, especially when agitated. This, in turn, has the potential to affect different disease pathogens in the manure.

Heat Treatment:

In a study done by Turner et al. in 1999, heat-treated swine manure between 50-60° C for five minutes with pH's ranging from 6.4 – 8.0. The more alkaline slurry required lower heat-treatment (50-55° C) and inactivated ASFV and SVDV (Swine Vesicular Disease Virus), while the more acidic slurry required a higher temperature heat-treatment (55-60° C) for the inactivation of SVDV, yet did not inactivate ASFV.

Similarly, Turner et al., 2000 demonstrated that FMDV (Foot and Mouth Disease Virus), PRV (Pseudorabies Virus), and CSFV (Classical Swine Fever Virus) were inactivated in pig slurry within three minutes when heated at high temperatures (67, 62, and 60° C).

Additionally, similar results from several pilot experiments allowed for recommendations to be made on a full-scale level, which included various heat-treatments to inactivate specific disease pathogens (60° C for ASFV, 65° C for PRV and SVDV, and 70° C for FMDV) for a minimum of five minutes. Although these are practices not commonly applied on farms, when producers are addressing disease outbreaks and the potential for spreading a foreign animal disease (FAD) these heating methods can be reviewed and adapted practices put into place to address health issues.

Additives:

Schmidt (2016), showed concentrations of PEDV in swine manure were inactivated with the addition of lime to achieve a pH of 10 for an incubation period of an hour. While this study was performed at lab-scale, it can be worked out that the amount of lime needed in 100,000 gallons of slurry is roughly 10,000 lbs. If possible to obtain lime in bulk quantities, perhaps lime could be a possible disease mitigation strategy based on this research.

Derbyshire and Brown (1979) demonstrated the possible use of calcium hydroxide as an additive to swine manure effectively increasing the pH to 11.5 and inactivating swine enterovirus and adenovirus. It is important to consider that this study is from 1979 and we now have different feed strategies and management that could affect the outcome differently today.

Potential:

While the above research trials show a viable way to potentially manage disease pathogens in manure, the feasibility of such treatments for on-farm use may be difficult. The ability to heat manure on farm with the specifications listed in the research could prove to be a daunting undertaking from an equipment and labor standpoint. The prospect of adding disease mitigating materials to slurry such as lime and calcium hydroxide may be attainable, however, the cost effectiveness would need to be considered.

Conclusions

Choose one or two biosecurity practices to improve upon immediately. Include other employees on the farm when developing and reviewing these practices. Stay-tuned for more information as we consider

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