

Detection and molecular characterization of swine hepatitis E virus in North Carolina swine herds and their faecal wastes

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ABSTRACT

Recent findings of almost genetically indistinguishable swine and human strains, have suggested swine play a role in the transmission of hepatitis E virus (HEV). The extent to which HEV may be present and persist in the faecal waste generated from intensive swine operations is largely unknown. The fate of swine waste liquid is often land application, possibly resulting in unintentional seepage into groundwater or run-off into surface waters, hence validating concerns of human exposure risks. Freshly passed swine faeces, barn flush liquid waste, and lagoon liquid from production sites in North Carolina were surveyed periodically for HEV using RT-PCR primers located in ORF2. On three farms where HEV RNA was detected in swine faeces, it was also found in stored liquid waste on several occasions. HEV presence was related to swine age but not to animal management and waste management procedures, which varied amongst the farms. Seasonal patterns of HEV prevalence could not be established as viral RNA was isolated at all time points from two farms. Phylogenetic analysis of 212 bases of the genomic RNA indicated that isolates resembled the known US swine and human strains (percentage nucleic acid homology 91 to 94%), with one amino acid substitution.

Key words | disease reservoirs, faecal contamination, hepatitis E virus, public health, water quality, zoonoses

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INTRODUCTION

Often characterized as an emerging pathogen, hepatitis E virus (HEV) is a known cause of epidemic and intermittent cases of enterically transmitted acute hepatitis. Notably, within developing countries, the majority of sporadic cases of viral hepatitis can be attributed to HEV and not the other major hepatotropic viruses (hepatitis A, B or C) (Emerson & Purcell 2003). Clinically, hepatitis E and hepatitis A infections are indistinguishable, with symptoms including jaundice, anorexia, nausea, vomiting and hepatomegaly. Yet, infection with HEV can be more severe, with mortality rates of about 1%; for pregnant women case-fatality rates can reach 27% (Kumar *et al.* 2004; Chau *et al.* 2006).

Epidemiological studies have established an association between HEV infected pregnant women and incidences of fatal fulminant hepatic failure (Jaiswal *et al.* 2001).

Cases of hepatitis E in developed countries, including the US, occur infrequently, although several seroprevalence studies report a 1–7% prevalence of HEV antibodies in individuals from countries where HEV is not endemic (Mast *et al.* 1997; Thomas *et al.* 1997; Meng *et al.* 2002; Gotanda *et al.* 2007). Recent reports suggest that cases of HEV in industrialized countries are autochthonous, largely overlooked, segregate into genotype III, and lack a precise source of infection (Mateos *et al.* 2006; Preiss *et al.* 2006;

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Gotanda *et al.* 2007; Dalton *et al.* 2007; Péron *et al.* 2007; Herremans *et al.* 2007).

Several possible animal reservoirs for HEV have been identified, but it is unclear what role they play in either human hepatitis cases or seroconversion in persons residing within non-endemic areas. Existing microbiological and epidemiological data suggest a potential role of swine in the human transmission of hepatitis E virus. Relevant findings include: global existence of anti-HEV seropositive swine (Meng 2003), genetic relatedness of swine and human isolates (Meng *et al.* 1997; Hsieh *et al.* 1999; Takahashi *et al.* 2003; Banks *et al.* 2004; Cooper *et al.* 2005), inter-species transmission of swine and human strains (Meng 2003; Meng *et al.* 1998a), and high seroprevalence levels amongst swine caretakers (Withers *et al.* 2002; Meng *et al.* 2002). A study of swine workers from North Carolina found nearly 11% (18/165) with evidence of HEV seropositivity (Withers *et al.* 2002). Moreover, direct evidence of zoonotic spread involving group consumption of undercooked wild boar meat has been documented (Tamada *et al.* 2004).

In the United States, a novel strain of swine HEV recovered from herds in the Midwest was found to have the greatest homology, at the nucleotide level, to the virus isolated from two human autochthonous clinical cases of hepatitis E (Meng *et al.* 1997). In a subsequent US study, 27 HEV isolates from 2- to 4-month-old swine from multiple farms in Iowa and Missouri were genetically characterized. The 348 nucleotide-long sequences most closely resembled only Genotype III strains (including other US human and swine isolates) and displayed little genetic diversity (0–12%). HEV RNA was detected in 35% (34/96) of the pigs and 20 of the 37 swine herds tested (Huang *et al.* 2002).

The extent to which HEV may be present in the faecal waste generated from intensive swine agriculture operations, as practised in North Carolina, is largely unknown. Typically, liquid swine waste does not undergo extensive treatment as is prescribed for human waste. Hence potentially little or no reduction of faecally shed HEV may occur during swine waste storage before the liquid waste is land applied. If infectious HEV is still present in land-applied swine waste, there is the potential for it to be transported off-farm via contaminated surface water, infiltrated groundwater or irrigated crops.

This study investigates swine HEV occurrence, presence and genetic characteristics in various swine waste samples from several North Carolina swine farms sampled over at least a one-year period. To gain a better understanding of virus occurrence and persistence in North Carolina swine waste management systems, freshly passed swine faeces, waste (manure) flushed from barns, and storage (lagoon and/or wetland) liquid was collected from each farm and evaluated by reverse-transcription PCR (RT-PCR) for the presence of HEV. HEV presence in these samples was examined in relation to characteristics of the swine farms and their swine populations, including animal age, as well as animal management and waste management procedures.

MATERIALS AND METHODS

Selection and survey of swine production facilities

A total of five farms in central and eastern North Carolina (NC) representing various swine production (growth) stages (feeder, feeder-to-finish and farrow-to-finish) agreed to participate.

Demographics of the farms surveyed are given in Table 1. In an 'all-in/all-out' process, animals are moved into and later out of areas (e.g. barns, rooms) as a group, while in a continuous management system, animals are moved into a confinement area intermittently, without respect to the duration of presence of other inhabitants. The weaning of piglets typically occurs after 21 days post-birth or at a weight of 20 kg. In a feeder production system, weaned piglets ('feeders') are produced and sold/raised off-site while a breeding stock of sows is retained. On a feeder-to-finish farm, feeder pigs are raised to market or slaughter weight (usually 100–120 kg). In contrast, a more traditional approach of farrow-to-finish encompasses all steps from breeding to finishing on the same farm.

All farms surveyed were active, had an on-site waste management system, and were either strictly commercial or university affiliated (educational) operations with more than 300 swine on the premises. Repeat sampling occurred for each of farms 1–4 on a quarterly basis to examine any seasonal fluctuations in prevalence over a period of at least one year. Seasons are defined as winter (December,

Table 1 | Demographics, animal husbandry, and waste management practices of farms 1–5

Farm information	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5
Farm type	University ~ 500	University ~ 600	Commercial	Commercial	Commercial
Number of swine (annual)			3,000 sows	1,800 sows	3,500
Production (growth) stages	Farrow to finish	Farrow to finish	Feeder	Feeder	Feeder to finish
Production system type	Variable*	Variable [†]	Continuous	All-in/all-out	Presumed continuous
Waste flushing system	Under-slats	Under-slats	Pit-recharge w/recycled lagoon liquid	Under-slats	Pit-recharge
Swine waste management system type	1-stage lagoon	Serial lagoons	2-stage lagoon	1-stage lagoon	Constructed wetland cells

*Nursery and farrowing rooms and finishing barn was all-in/all-out, breeding and gestation barns were continuous.

[†]Nursery and farrowing rooms were all-in/all-out, breeding and finishing barns continuous.

January, February; average monthly mean temperature 7.3°C), spring (March, April, May; average monthly mean temperature 16.8°C), summer (June, July, August; average monthly mean temperature 25.8°C) and fall (September, October, November; average monthly mean temperature 17.6°C), based on North America as the reference point for climate. Samples from farm 5 were only obtained at three time points due to availability limitations.

Information obtained included: production stages present, frequency of barn cleaning and type and operating conditions of the swine waste management system.

Sample collection

A total of 10 individual faecal samples of approximately 10 g each were gathered from different locations (pens) in each barn. Other samples collected from the production site included barn flush (faeces plus urine mixed with wash and flush water) and treated/stored liquid waste. Approximately 2 l of barn flush liquid (including its waste solids) and 4 l of liquid waste from lagoons or alternative treatment/storage systems (e.g. constructed wetlands) were collected. All samples were kept cool with ice packs or stored at –20°C for later processing and analysis.

Virus recovery, extraction and concentration

The method for recovery, concentration and purification of HEV in swine wastes was adapted from methods previously developed to detect enteric viruses in municipal and airline sewage (Shieh *et al.* 1997). This method was employed given

the potentially low levels of viruses in sewage and similar liquid waste with high concentrations of suspended solids. Initially faecal samples were diluted in sterile phosphate buffered saline as 10% (w/v) faecal suspensions (w/v). For virus recovery, swine faeces (10% w/v suspension), fresh barn flush wastes and lagoon liquid samples with high suspended solids concentrations were separated into liquid and solid fractions by centrifugation at 5,000 × g for 30 minutes at 4°C. Sedimented solid fractions were resuspended in small volumes of 3% beef extract (BE) and subjected to solvent (chloroform) extraction. After centrifuging, the resulting supernatant was recovered and added to the waste supernatant from initial sample centrifugation. Extraction of the solid material with 3% BE and solvent was repeated as described. Viruses were further concentrated and purified from the combined supernatants by polyethylene glycol (PEG) precipitation overnight at 4°C (PEG concentration = 8%, added NaCl = 0.3M). Samples that did not contain high concentrations of suspended solids (e.g. swine waste liquid) were subjected to PEG precipitation without initial solids separation by centrifugation and BE + solvent extraction. In general, the liquid waste from lagoons contained enough solids to warrant centrifugation and extraction. After centrifugation, PEG precipitates were resuspended in a small (typically 1/100th of the original sample) volume of pH 8.0, 20 mM Tris – 0.2% Tween 20 buffer solution. After an equal volume of chloroform was added, the sample was vortexed and centrifuged at 5,000 × g for 15 minutes at 4°C. The supernatant was decanted and saved while a volume of pH 8.0, 20 mM Tris – 0.2% Tween 20 buffer solution equal to one-half of the original volume of

resuspended PEG precipitate was added to the remaining solids and chloroform. This mixture was emulsified by vortex mixing and centrifuged ($5,000 \times g$ for 15 minutes at 4°C). The resulting supernatant was combined with the initial supernatant, and an aliquot (140 to $420 \mu\text{l}$) of this sample was subjected to the QiaAmp Viral RNA kit (Qiagen Inc.; Valencia, California) following recommended kit procedures. The remaining supernatant was stored at -70°C .

Virus detection by RT-PCR

Primer selection

Primers used for initial screenings of all samples are specific for the US swine HEV and are located in the open reading frame 2 (ORF2) capsid protein region of the viral genome (primer set 1; Table 2). They were applied previously in the detection of the novel US swine HEV strain (Meng *et al.* 1998b). Because the nucleic acid sequence of any swine HEV could differ significantly from previously reported strains, samples were also screened with additional HEV primer sets. The nucleotide sequences of 20 full-length HEV genomes encompassing all genotypes were aligned. Regions for all three open reading frames showing the greatest homology were examined and primer sets were selected (primer set 2; Table 2).

Two-step RT-PCR

For the initial screening of all samples, primer set 1 (Table 2) was used in a two-step RT-PCR protocol which was found to produce the greatest sensitivity in terms of lower level of

detection by endpoint titration (J.A. Kase, unpublished data). All two-step RT-PCR reagents were from the Gene Amp RNA PCR kit (Applied Biosystems; Foster City, California) with previously described initial RT-PCR conditions and those recommended by the manufacturer (Meng *et al.* 1998b). The subsequent nested PCR assay used $10 \mu\text{l}$ of the first round PCR product and 40 cycles consisting of: 1 minute at 94°C , 1 minute at 52°C , and 2 minutes at 72°C . Positive (e.g. US swine HEV) and negative controls were included.

One-step RT-PCR

For a second screening of selected samples, primer set 2 (Table 2) was used with a one-step RT-PCR kit (Qiagen Inc.; Valencia, California) according to manufacturer's instructions. This method produced the most sensitivity using this particular primer pair (J.A. Kase, unpublished data) with cycling conditions consisting of 50°C for 30 minutes, 95°C for 15 minutes and 40 cycles consisting of 1 minute each at 94°C , 52°C , and 72°C , with a 10 minute final step at 72°C . A second round of amplification followed with the internal primer pair and amplification conditions. Negative and positive (e.g. US swine HEV) controls were included.

Sequence analysis of PCR amplicons

Nucleic acid sequencing of probable HEV amplicons was done through the University of North Carolina (UNC-CH) Automated DNA Sequencing Facility. In preparation for sequencing, PCR reactions were subjected to the QIAquick PCR purification kit (Qiagen Inc.; Valencia, California).

Table 2 | Primers used in PCR assays

	Primer name	Orientation	Amplicon size*	Nucleotide sequence (5' to 3')
Primer set 1	SHEVFEXT	Sense	429 bp	AGCTCCTGTACCTGATGTTGACTC
	sHEVREXT	Anti-sense	(5526–5955)	CTACAGAGCGCCAGCCCTTGATTGC
	sHEVFINT	Sense	288 bp	GCTCACGTCATCTGTCGCTGCTGG
	sHEVRINT	Anti-sense	(5596–5884)	GGGCTGAACCAAAATCCTGACATC
Primer set 2	JAKFExt	Sense	508 bp	ACAGAATTGATTTTCGTCGGC
	JAKRExt	Anti-sense	(6323–6831)	TTAGTK(G/T)GTR(G/A)CCW(A/T)GCCTCCC
	JAKFInt	Sense	127 bp	GTY(C/T)GTCTCR(G/A)GCCAATGGC
	JAKRInt	Anti-sense	(6371–6498)	TAATCCTGR(G/A)ATAACY(C/T)ACACG

*Sequence position relative to US swine HEV given in parentheses.

DNA was sequenced at the facility on a Model 377 DNA Sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, California) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer, Applied Biosystems Division, Foster City, California). All resulting sequencing information was compared with every full-length HEV genomic sequence available through the GenBank database (National Center for Biotechnology Information, National Institutes of Health, <http://www.ncbi.nlm.nih.gov>, for confirmation of identity). Evolutionary distance trees were constructed using TREECON for Windows, version 1.3b (Van de Peer & De Wachter 1994). Distance estimation analysis was conducted by either the Jukes & Cantor (1969) or Saitou & Nei (1987) method based on a 179 nucleotide region of ORF2. Trees were constructed using the neighbour-joining method and any difference in resulting phylogenetic tree construction using the two methods of distance estimation was noted. Bootstrap values, providing validity for a particular constructed tree, were generated based on 1,000 resamplings of the data set. Both single-sequence rooted and unrooted trees were constructed for comparison.

RESULTS

Samples collected from all farms were processed and analysed for the presence or absence of HEV RNA by PCR using the described methods. Overall, HEV was not detected in any sample at any survey time point for farms 1 and 4. On the other hand, using primer set 1, at least one sample from farms 2 and 5 contained detectable amounts of HEV RNA at every sampling. HEV RNA was detected at farm 3, with more variability in occurrence than at farms 2 and 5. In addition, certain selected samples from a farm in which HEV RNA was not detected and samples from a farm in which HEV RNA was detected in some but not all of the samples were further screened using primer set 2; no new positive samples were identified. Tables 3 and 4 present results for farms 2, 3 and 5.

Farm 1

Farm 1 is a university-affiliated farrow-to-finish farm (Table 1). Populations of swine are transient and dependent mainly on

current academic research needs (e.g. feed formalization and utilization studies). Faeces were collected from individual rooms (nursery and farrowing barns) and on a per barn basis (finishing, gestation and breeding barns). Overall, the residency flow for the nursery and farrowing rooms and finishing barn was all-in/all-out with entire barns or rooms cleaned and disinfected prior to the next rotation. A thorough cleaning was completed less frequently for the breeding and gestation barns (continuous type of production system). Once a week, efforts were made to spray down each area with water.

Waste generated inside the barns is allowed to fall through the slotted floor to a gutter system that uses recycled lagoon liquid to flush wastes. The wastes are flushed every four hours and are transported through underground pipes to a holding area where solids are separated from the liquid fraction prior to liquid entry into the lagoon. Samples of barn flush were taken prior to solids separation and also a portion of the solid material was obtained. The barn(s) from which the barn flush originated at the time of each sample collection is unknown so the samples may represent a composite of more than one barn. Liquid swine waste is managed through the use of open, ambient-temperature facultative (upper layer aerobic and lower layer anaerobic) lagoons and periodically this material is sprayed onto adjacent fields. Lagoon influent was collected where the barn flush liquid enters the lagoon and lagoon effluent was obtained from the opposite end of the lagoon where pumping occurs for spray irrigation onto adjacent land.

HEV RNA was not detected in any sample collected during five time points (winter (twice), spring, summer and fall). In addition, samples collected in preliminary work from farm 1 during the previous year (winter and spring), were also not found to have detectable HEV genomic RNA (data not shown). To examine whether positive samples could not be detected in the initial screening due to the primer set and RT-PCR conditions used, selected samples were examined with primer set 2 and the alternative RT-PCR amplification method. Negative results for HEV were confirmed by these analyses.

Farm 2

Farm 2 is a smaller, university-affiliated farrow-to-finish farm (Table 1). The smaller farm size permitted freshly passed faeces

Table 3 | Farm 2 and 3 individual samples positive for HEV

Farm	Season	Faecal composite samples	Liquid faecal waste	
2	Spring	Finishing barn (~3 month old) 2A*	Lagoon liquid 2B*	
	Summer	Nursery barn (12–13 week old) 2C*	–	
	Fall	Nursery barn (11 week old)	Lagoon 1 liquid	
		Finishing barn (age unknown)	Lagoon 3 liquid	
Winter	Finishing barn (~3 month old)	–	Barn flush (finishing)	
		–	Lagoon 1 liquid	
3	Winter 1	–	Lagoon 3 liquid	
			Barn flush (gestation)	
			Lagoon influent	
	Winter 2	Farrow barn 3A*	–	Lagoon effluent
			–	Barn flush (gestation) 3B*
			–	Barn flush (finishing) 3C*
			–	Lagoon influent 3D*
	Spring	–	–	Lagoon effluent 3E*
			–	Barn flush (gestation) 3F*
	Winter 3	–	–	Lagoon influent 3G*
–			Lagoon effluent	

*Identity of PCR amplicon confirmed as HEV by nucleic acid sequencing (Figure 1).

Table 4 | Farm 5 detailed sample descriptions and PCR results

Sample description	PCR result		
	Spring	Winter 1	Winter 2
Barn 1 (composite faecal sample)	P (5D)*	P	P
Barn 2 (composite faecal sample)	P (5E)*		
Barn 3 (composite faecal sample)	N		
Barn 4 (composite faecal sample)	P		
Sludge from barn pit	P (5A)*	No collection	No collection
Liquid from barn pit	P (5C)*	P	P
Solids (separator)	P (5B)*	P	P
Outer wetland cell influent	P (5H)*	P	N
Outer wetland cell effluent	N	N	N
Inner wetland cell influent	P (5F)*	P	P
Inner wetland cell effluent	P (5G)*	P	N
Storage pond (4 litres)	N	N	N

*Identity of PCR amplicon confirmed as HEV by nucleic acid sequencing (Figure 1).

N—HEV RNA was not detected in sample tested.

P—HEV RNA was detected in sample tested.

to be collected from individual pens (nursery, farrowing and finishing) and on a per barn basis (gestation/breeding barn). Overall, the residency flow was all-in/all-out in nursery and farrowing areas, with the entire barn or room cleaned and disinfected prior to the next rotation. On a weekly basis, the floors of all barns were power washed with only water. Finishing and breeding areas were never disinfected, but the crates were power washed with water when empty.

Waste generated inside the barns falls through the slotted floor to a gutter system that uses recycled lagoon liquid to flush wastes. Barn wastes are flushed every four hours and are transported through underground pipes to a lagoon.

Liquid swine waste is managed through a series of three open, ambient temperature facultative lagoons and periodically this material is sprayed onto adjacent fields. Waste travels from lagoon 1 to lagoon 2 and then through a wetland area where runoff enters lagoon 3. Water from lagoon 3 is periodically used both for spray irrigation onto adjacent fields and as a source of water for barn flushing. Barn flush and lagoon liquid samples (lagoons 1 and 3) were collected at all time points.

As presented in Table 3, HEV RNA was detected in several faecal and liquid samples collected. Although many of the faecal samples tested were negative for HEV genomic RNA, those coming from swine approximately 3 months of age were positive with one exception (winter-finishing barn). In general, swine less than 2.5 months and those four months or older were not found to be excreting detectable amounts of HEV.

Liquid waste samples obtained from farm 2 were also positive for HEV RNA except for the summer survey samples (Table 3). HEV genomic viral amplicons were detected in lagoon liquid samples obtained during the spring, fall and winter. However, all barn flush samples except for one were negative for HEV RNA; a winter sample from the finishing barn was HEV-positive. Based on comparison to known, published sequence information for other HEV strains, HEV RNA presence was confirmed in samples from the spring and summer surveys (Table 3, Figure 1).

Farm 3

Farm 3 is a commercial feeder production farm where piglets are weaned at age 21 days and shipped off site (Table 1).

Swine are housed within six separate barns: four gestations and two farrowing. Composite faecal samples were collected by farm personnel from each barn at each sampling time point. Residency flow was continuous and the barns were periodically cleaned as the sows were moved into the farrowing houses.

Each day, one of eight pits within each barn was flushed with recycled lagoon liquid. At each sampling time point, a barn flush sample was obtained from both a gestation and farrowing barn. The origin of the barn flush (i.e. gestation barn #) varied depending on the timing of farm visit.

On farm 3, barn manure wastes are flushed into a first-stage, covered, in-ground ambient temperature anaerobic digester. The effluent from this digester flows into a second-stage open lagoon that is probably facultative. Liquid from this second lagoon is land applied by spray irrigation. Samples were taken both at the point where liquid effluent from the digester enters the second lagoon and at the opposite end of the lagoon where liquid is pumped for land application.

Of faecal samples collected on six different sampling dates, only one faecal sample taken from a farrowing barn during the second winter survey was positive for swine HEV (sHEV) RNA by PCR analysis and nucleic acid sequencing (Table 3).

In contrast, sHEV RNA was detected in barn flush samples collected during two separate winter visits and a spring visit. In the case of winter (visit 2), both farrow and gestation barn flush samples had detectable genomic sHEV.

HEV genomic RNA was detected in lagoon samples taken at the same time points as positive barn flush samples (Table 3). In addition, a lagoon sample taken during the winter 3 survey contained detectable sHEV RNA. However, it was only during the winter 2 survey that samples from both lagoons 1 and 2 were HEV-positive. Nucleic acid sequencing confirmed the identity of probable HEV amplicons in the farrow barn faecal sample from the winter 2 survey and liquid waste samples of the winter 2 and spring surveys (Figure 1). A second screen with primer set 2 did not detect HEV RNA in any of the faecal samples but confirmed the presence of viral material in three out of four liquid waste samples previously found positive (data not shown).

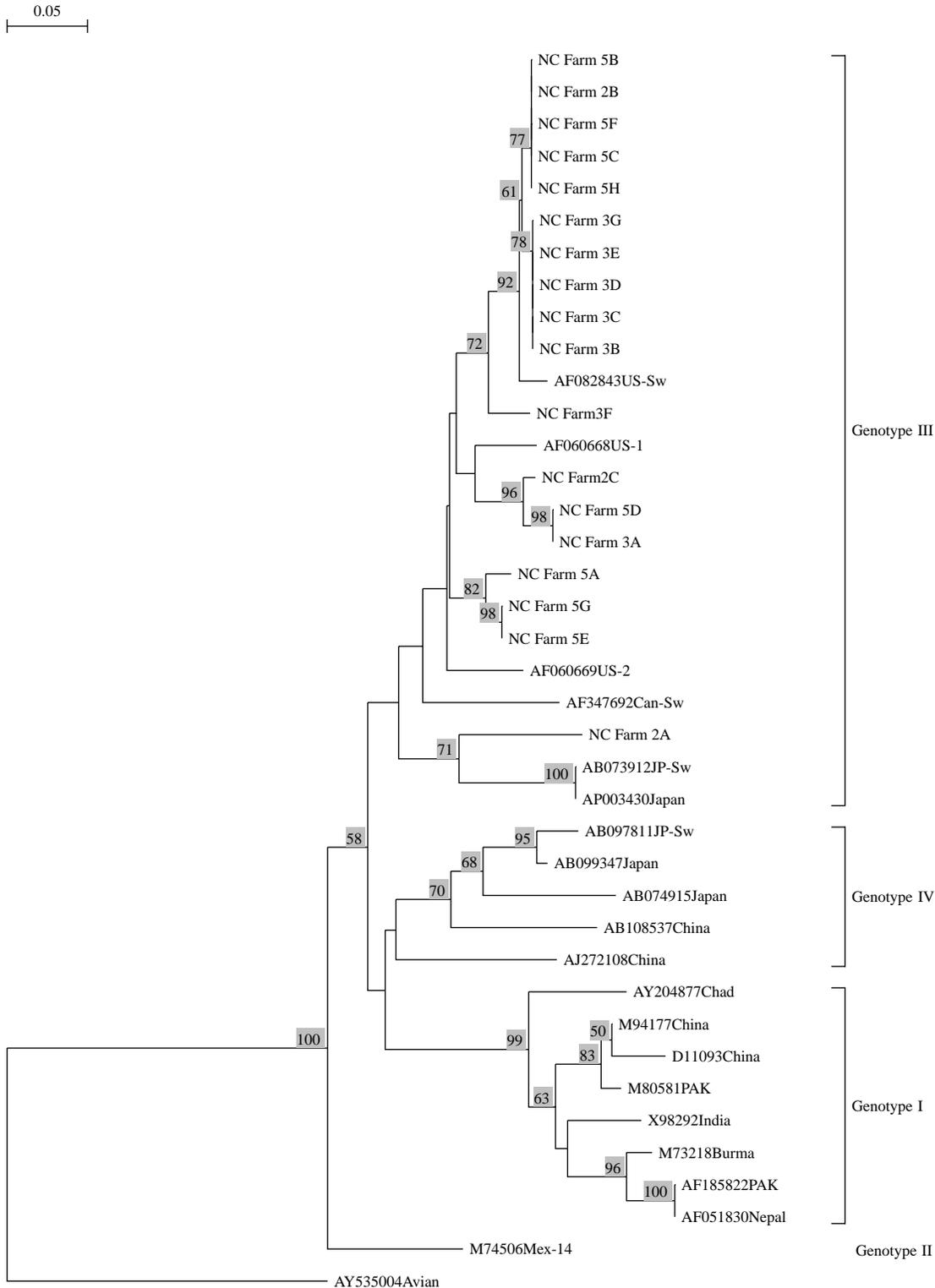


Figure 1 | Dendrogram showing the evolutionary relationship between the SHEV of North Carolina swine and other published human and swine nucleic acid sequences. Phylogenetic analysis on 179 nucleotides was executed using TREECON for Windows computer software (Van de Peer & De Wachter 1994) with Jukes and Cantor correction for evolutionary rate. Bootstrap analyses were conducted and percentages are shown at the respective nodes. GenBank accession numbers are given for reference.

Farm 4

Like farm 3, farm 4 is also a commercial feeder production facility (Table 1). Swine are housed within seven separate barns: three gestations, three farrowing and one breeding. Farm personnel collected composite faecal samples from each barn, barn flush and lagoon liquid at each sampling time point. Residency flow was all in/all out, and the barns were thoroughly cleaned as groups of animals are rotated out (every 19–21 days). The breeding barn was cleaned on a weekly basis. For farrowing and gestation barns, sections were cleaned weekly as they were emptied and prior to the introduction of a new group. Liquid faecal waste was managed with a single ambient temperature anaerobic lagoon.

All liquid waste samples (i.e. barn flush collected from gestation, breeding and farrow barns, and lagoon liquid) were negative for HEV RNA. The samples designated as barn flush collected at this particular farm differ from those collected at the other farms. In this case, the flush material collected was a portion of the recycled lagoon liquid used to do the flushing of waste material from the barns not the resulting waste stream generated after barn flushing.

Collected faecal samples included separate composite samples from three gestation, three farrowing and one breeding barn. HEV RNA was not detected in any sample. Moreover, faecal and liquid waste samples collected previously during preliminary studies also were found not to contain detectable HEV RNA (data not shown).

Selected samples, specifically those from the spring and fall samplings, were further screened with primer set 2 and confirmed previous positive results.

Farm 5

Farm 5 was a commercial feeder-to-finish farm where swine were housed in four barns (880/house) and stand on slatted floors (Table 1). A composite faecal sample was obtained from only one barn unless otherwise noted. Waste material was allowed to fall between the slats into a pit below, which was emptied on a weekly basis and recharged with water from an on-site holding pond. The holding pond stored the liquid effluent of a constructed wetland used to treat solids-separated liquid waste. Houses were power washed with

plain water between rotations. Waste generated from the pit-recharge system was managed through the use of a commercial solids separator/constructed wetlands system. Following passage through the solids separator, the liquid portion entered either the outer or inner wetland cell for biological treatment before entering a treated wastewater holding pond. Farm personnel collected faecal and liquid waste samples at intervals convenient for unrelated research.

Faeces, barn flush and wetland liquid samples were positive for the presence of HEV RNA regardless of season (Table 4). Genomic HEV RNA was detected in both the inner and outer wetland cells influent liquid. However, only the inner wetland cell effluent was shown to contain HEV compared with the outer wetland cell effluent (Table 4, all three time-points). HEV RNA was not detected in wetland effluent holding pond liquid samples taken during any of the survey times (Table 4).

The alignment of approximately 222 nucleotides obtained from genetic sequencing of several North Carolina (NC) HEV positive samples (farms 2, 3 and 5) indicate considerable similarities with genotype III strains. Although several nucleotide variations were observed, only one difference represented an amino acid change. Percentage nucleotide homology between selected NC HEV isolates and other previously characterized HEV strains was calculated. For maximum contrast, NC isolates found in separate groupings as indicated in Figure 1 were chosen. Intra-farm (farm 5) diversity was 7.7% (NC5D and NC5E) while inter-farm diversity (farms 2, 3, 5) ranged from 7.8% (NC3D vs. NC5E) to 14.7% (NC2A vs. NC3D and NC2A vs. NC5E). NC farm isolates demonstrated 84.8–98.2% nucleotide identity with the swine and human US isolates compared with 81.2–90.0% and 82.9–89.0% identity with the Canadian and Japanese swine isolates, respectively. Interestingly, the NC isolates more closely resemble other swine and human HEV strains than they do each other. As an example, the percentage identity between NC3D and US sHEV was 98.2% compared with 85.3–92.2% homology with other NC swine isolates.

Phylogenetic analysis on a 179 nucleotide locus for all 18 NC farm isolates and other HEV strains revealed several distinct groupings as seen in Figure 1. Interestingly, the NC isolates appear throughout the Genotype III grouping, with

isolates from the same farm appearing in different clusters. Repeated analysis was conducted with the same genetic information using several of the available models (e.g. [Saitou & Nei 1987](#)). None resulted in a radically different dendrogram.

DISCUSSION

Political pressure has encouraged North Carolina farms to consider progressive alternative waste management strategies for the massive amounts of faecal waste produced from intensive swine facilities that, until now, have not been examined for their influence on sHEV prevalence. Furthermore, continued media attention and proposed stricter industry regulation illustrate the need for sound scientific data for policy making regarding potential public health and environmental impacts.

In this study, five swine production operations were surveyed and HEV viral RNA was detected in swine waste collected from three farms ([Tables 3 and 4](#)). A similar study, but focused only on Midwestern farms, found sHEV in a majority of faecal slurry material but in a minority of the eight lagoons tested ([Kasorndorkbua *et al.* 2005](#)). Since farms were only visited once, seasonality was not assessed in that study. Pigs from six farms tested positive for sHEV but a relatively low number of individual swine from each farm were sampled. Infectivity of recovered virus from a manure pit was documented in two out of three specific pathogen-free pigs by intravenous inoculation but not by oral inoculation. Notably, one pig did seroconvert to HEV following intravenous inoculation with lagoon inoculum but did not exhibit viral faecal shedding.

To examine seasonal patterns of HEV prevalence, repeat farm sampling was done in different seasons of the year in this study. Viral genomic HEV material was detected at all seasonal sampling time points from farms 2 and 5 ([Tables 3 and 4](#)). These results suggest little seasonal influence or perhaps the carry over of virus from season to season. On farm 3, only one faecal sample (farrowing barn) was ever found to be positive for HEV out of the 36 samples analysed. However, liquid swine waste samples from both spring and winter sampling were positive for HEV ([Table 3](#)), indicative of the periodic presence of sHEV

in the animals, but more consistent presence of sHEV in the accumulated swine waste.

In general, on farm 2, swine less than 2.5 months and those four months or older were not excreting detectable amounts of HEV ([Table 3](#)). These findings for age-related sHEV presence in the animals are consistent with previous studies, as swine are believed to become susceptible to HE infection following a waning of any protective maternal antibodies ([Meng *et al.* 1997](#); [Wu *et al.* 2002](#)) and the majority of adult swine have anti-HEV IgG antibodies, suggesting previous infection that occurred some time ago ([Meng *et al.* 1997](#)). One unexpected finding was that none of the swine in the probable susceptible age group from the other academic farm surveyed, farm 1, was found to be excreting detectable amounts of sHEV. The reasons for the presence of sHEV in juvenile swine of about the same age on one farm but not another cannot be explained from the limited sampling of this study.

The extent to which swine management and cleaning regimes influence HEV presence in swine faeces and waste is uncertain from the results of this study. There were no major differences in such practices between the two academic farms (farms 1 and 2), yet considerable disparity in their HEV prevalence. One possible explanation for the consistent presence of HEV on farm 2 may be that the source of water for barn flushing is liquid from lagoon 3 which has been shown to contain HEV RNA ([Table 3](#)). Because flushing of the waste with recycled lagoon liquid occurs under the slatted floor, it is possible for infectious viral particles to be released from the liquid as droplets and aerosols and perhaps be transmitted to new susceptible animals. Under these conditions, cleaning and disinfection practices would have little impact on sHEV infection prevention and control. If lagoon liquid from a farm can carry infectious HEV into the barns, it might explain why infected animals were observed in a barn disinfected regularly (nursery) as well as in one never disinfected (finishing).

Although the farm 1 source of flushing liquid is not clear, none of the samples taken from the lagoon was ever found to contain detectable amounts of HEV, thus making it an unlikely source of contamination of the barn environment or its swine populations. However on farm 3, HEV was found in lagoon liquid only once, which

corresponded to the presence of HEV RNA in swine faeces and therefore infection (Table 3). This particular farm is atypical because of its use of bio-filtered lagoon liquid for barn flushing. Through the use of a submersible pump, located in the second-stage lagoon near the inlet of waste from the first-stage lagoon, liquid is removed, pumped through a bio-filter unit, and stored in a holding container. If infectious HEV was in the original pumped material, it is possible that reduction in titre or viral inactivation took place.

Similar to farm 2, sHEV RNA was detected at all sampling time points from farm 5 (Table 4). Moreover, sHEV RNA could be detected in all samples (solid and liquid) except for the liquid coming from the storage pond. The extent of positivity seen on this particular farm may be explained by the age of swine present. The absence of HEV in the holding pond might be due to its appreciable physical removal, inactivation and dilution occurring in the wetland cells and the pond itself. Biological activity in the wetland may contribute to virus inactivation by removing or damaging the virions and their RNA before discharge of the liquid waste into this holding pond. Previous studies have shown that the bacterial activity of aerobic biological processes, such as those occurring in activated sludge treatment and in surface waters, cause virus inactivation (Ward 1982; Ward *et al.* 1986).

Only spring and winter sampling of farm 3 indicated the presence of HEV RNA (Table 3). Interestingly, we found just one positive faecal sample (farrowing barn) despite the detection of viral material in liquid waste samples. Short-term or intermittent shedding of HEV in faeces may introduce contamination into the lagoon(s) where it may persist for considerable periods of time. Unlike most NC commercial swine farms that utilize only a single anaerobic lagoon for liquid waste storage, this farm pumps barn flush into a covered anaerobic digester and then discharges digester effluent into the larger aerobic lagoon. Since HEV RNA was detected on three separate occasions in samples from the second-stage lagoon it does not appear that all viruses are associated with particles settling in the first-stage covered anaerobic digester. Moreover, because liquid from the second-stage lagoon is being used for crop and greenhouse produce irrigation, potential public health implications exist. Appreciable inactivation of HEV might be

expected given treatment in the two biological processes of anaerobic digestion followed by lagoon treatment and storage. It does appear that lagoon storage on conventional Midwestern US farms may not necessary lead to a loss of infectivity (Kasorndorkbua *et al.* 2005). However, without experimental evidence showing the extent of HEV reduction in each treatment step on a quantitative basis, the extent of HEV reduction through the treatment system cannot be known.

Following genetic analysis, all NC isolates were found to be similar but distinct from other previously characterized HEV strains. Reports from parts of Asia, Europe, Canada and the US indicate substantial genetic diversity amongst sHEV strains. In many cases, sHEV strains most closely resemble indigenous human strains in the same country or geographic area rather than other characterized swine strains from different countries or geographic areas (Meng *et al.* 1997; Hsieh *et al.* 1999; Takahashi *et al.* 2003; Banks *et al.* 2004; Cooper *et al.* 2005). Regardless of geographic location, swine HEV strains phylogenetically group as Genotype III or IV rather than representing all of the four human HEV genotypes. NC isolates most closely resembled other US HEV isolates (Genotype III) as shown in Figure 1.

Farm 3 and farm 5 isolates demonstrated approximately 91–98% nucleotide identity with the swine and human US isolates compared with about 83–92% identity with the Canada, Japan and Costa Rica swine strains. The genetic diversity among these three NC isolates was about 8%. Huang *et al.* (2002) found similar results in a study conducted on swine herds from Iowa and Missouri. Nucleotide similarities among these strains and the previously characterized US human and swine strains were 89–98%; while diversity amongst the strains ranged from 0 to 12%. However, an isolate from farm 2 most closely resembled the Japan sHEV strain (89% nucleotide identity) rather than the North American strains, including other NC isolates.

Phylogenetic analysis revealed isolates from the three NC farms sorting into three different groups (Figure 1). However, only 179 nucleotides were available for comparisons and bootstrap values below 50% for several of the branch configurations suggest that the topology may lack robustness. Genotyping based on short nucleotide

sequences is conceivably not as conclusive as analysis based on genome-length sequences. However, [Schlauder & Mushahwar \(2001\)](#) demonstrated, using a 287 base fragment of ORF 1, that it was possible to group HEV strains available at the time into four major genotypes and as many as nine sub-groups that, years later, are largely supported by the rigorous statistical analysis done by [Zhai *et al.* \(2006\)](#). Notably, the largest cluster of farm strains almost exclusively consists of isolates from liquid swine waste as opposed to solid faecal material. The significance of this finding is not clear. One exception is isolate 2A, which resembles the strain isolated from Japan swine and had one amino acid substitution. Numerous nucleotide changes were detected in the other NC isolates. This finding is consistent with previous evidence for a close resemblance between sHEV found in Midwest US swine and the US strains of human HEV ([Huang *et al.* 2002](#); [Kasorndorkbua *et al.* 2005](#)). Isolates were examined in an area of ORF2 approximately 100 base pairs upstream from the area of the HEV genome amplified in this study. Direct comparison with other newly recognized genotype III strains, most notably from several European countries, is not possible due to the lack of overlapping amplified region of the HEV genome ([Banks *et al.* 2004](#); [Preiss *et al.* 2006](#); [Herremans *et al.* 2007](#); [Fernández-Barredo *et al.* 2007](#)). However, given the close genetic similarities between the strains described in Europe and those previously identified in the US, by extrapolation, it would be reasonable to suggest a similar homology with the strains reported here. Full genome HEV sequences from countries outside of the US and Asia are lacking.

Water quality concerns about HEV exposure and HE transmission arise when drinking and recreational waters become contaminated with human and animal wastes. Outbreaks of waterborne HE from faecally contaminated water have been previously documented in developing countries ([Krawczynski 1993](#)). In Europe and the United States, HEV has been previously recovered from municipal sewage ([Clemente-Casares *et al.* 2003](#)) with presumably infectious virus found in raw sewage from Barcelona, Spain ([Pina *et al.* 1998](#)). Furthermore, experimental evidence now exists suggesting that the virus is not as heat labile as previously thought in faecal suspensions ([Emerson & Purcell 2005](#)) or in undercooked pig parts ([Feagins *et al.*](#)

[2008](#)). Also, food safety concerns occur when human and animal agricultural waste is used for irrigation of produce such as tomatoes and strawberries that are likely to be eaten raw and possibly without washing or when such waste contaminates shellfishing waters. There is only limited epidemiological evidence of a causal connection between environmental waterborne exposures to swine waste and domestically acquired sporadic cases of HE in developed countries such as the UK, US and the Netherlands ([Banks *et al.* 2004](#); [Sadler *et al.* 2006](#); [Amon *et al.* 2006](#); [Herremans *et al.* 2007](#)). However, the possibility that swine and their wastes may play a role in human exposure and infection is supported by evidence of nearly identical genomic HEV sequences in swine and humans in these geographical locations ([Banks *et al.* 2004](#); [Amon *et al.* 2006](#)), experimental cross-species transmission of both swine and human HEV isolates, and evidence implicating shellfish as a food-borne source of infection for two of the eight cases of HE identified in the UK in 2005 ([Sadler *et al.* 2006](#)).

CONCLUSION

The results of this study provide evidence that sHEV is endemic within swine populations in North Carolina. To our knowledge this is the first study to consider how farm-related variables (i.e. swine production system and waste management system) might influence the prevalence and persistence of HE infections and sHEV presence in swine faeces and collected swine wastes. North Carolina swine farms represent the growing trend in the swine industry of using separate farms for swine of different age groups, along with more progressive and effective waste management strategies. Although rigorous quantitative analysis was not possible due to the small number of farms represented and viability of recovered virus was not addressed, this study provides preliminary evidence of sHEV presence and persistence in treated and stored swine wastes as well as fresh faeces.

On all three North Carolina farms where HEV RNA was detected in swine faeces, it was also found in stored liquid waste on several occasions over the duration of the study. Such liquid waste is often land applied, creating the potential for unintentional seepage into groundwater or

run-off into surface waters, including waters used for recreational bathing and shellfishing, and possible contamination of produce by irrigation with HEV-contaminated water or waste, all of which create risks for human exposure.

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