Evaluation of a non-invasive screening approach to determine hepatitis E virus status of pig farms

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Abstract

Background Identifying pig farms infected with hepatitis E virus (HEV) is a key aspect to implement surveillance programmes for this emerging zoonotic agent. Detection of HEV in blood has several drawbacks, including animal handling, economic costs and animal stress. The objective of this study was to evaluate the effectiveness of a non-invasive screening approach for determining the HEV status of pig farms under different management systems.

Methods Forty stool samples randomly collected from the pen floor of 17 intensive pig farms and the yard of nine extensive ones were tested for HEV RNA. The invasive method used to confirm the HEV status of the farm was HEV RNA analysis of serum samples randomly collected from 40 animals on each farm.

Results Twenty-one HEV-positive farms were detected by invasive and non-invasive methods. No positive serum or stool samples were detected on five intensive farms. A high intertest agreement (K=1; P<0.00001) was observed between both methodologies, showing the stool screening approach a 100 per cent of sensitivity and specificity with respect to the invasive method. Likewise, a significant negative relationship was observed between the HEV within-farm prevalence and the number of the first HEV-positive stool sample found (Spearman's rho=−0.64; P=0.0004). This negative relationship was higher in intensively managed farms.

Conclusion This non-invasive screening approach could be reliably applied in a large-scale surveillance programme for determining the HEV status of pig farms under different management systems.

Introduction

Hepatitis E virus (HEV) is the causative agent of hepatitis E in people, one of the most common causes of acute hepatitis worldwide, and is also able to infect many animal species.[1 2] The clinical manifestations of HEV infection are very variable, ranging from subclinical to symptomatic (sometimes severe) forms. Acute HEV infection may adversely affect the clinical course of some comorbidities, as is the case of chronic liver disease, where it is associated with a higher rate of liver decompensation and death, or in immunosuppressed patients, where the disease can evolve into chronic forms with rapid progression to liver fibrosis and development of cirrhosis and terminal liver disease.[2–4]

HEV is classified under the genus *Hepevirus* and family Hepeviridae, and has a positive-sense, single-stranded RNA genome.[5] The definition of HEV subtype reference strains established a set of whole genome reference sequences for HEV-1 to HEV-8 subtypes of this genus.[5–7]

In Europe, the most common genotype of the virus is genotype 3, and pigs are considered the main source of zoonotic transmission of HEV through consumption of raw or undercooked pork products.[5 8–10] Transmission may be favoured by the widespread distribution and high prevalence of HEV infection on European pig farms, with farm-scale prevalence of up to 31 per cent

Veterinary Record (2020) doi: 10.1136/vr.105840

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Provenance and peer review Not commissioned; externally peer reviewed.

Received December 28, 2019
Revised March 17, 2020
Accepted May 4, 2020

reported in Italy,[11] 34.1 per cent in Canada,[12] 37.5 per cent in Norway,[13] 47.2 per cent in Spain,[14] 63 per cent in Finland,[15] and 100 per cent in the UK[16] and Portugal.[17] Furthermore, different studies suggest that the type of pig management system could influence HEV prevalence, having found significantly higher viral circulation in extensively reared pigs than in those bred under intensive farming conditions.[18 19] In Spain, a country where the pig production system accounts for 37 per cent of the total livestock produced and around 10 per cent of pig production is carried out in extensive management systems,[20] the disease magnitude could be underestimated due to the absence of HEV monitoring programmes.[10 21]

Actually, hepatitis E is not included in the list of Notifiable Diseases of the World Organisation for Animal Health (OIE), and to the authors' knowledge uninterrupted surveillance programme of the pig reservoir has never been implemented in any country. Because of its implications for public health, it is important to determine the presence of HEV on pig farms in order to implement HEV surveillance programmes, gather information about the occurrence and diversity of circulating strains, and establish control measures to reduce the risk of HEV infection and transmission.[9 22]

Several molecular and serological methods have been developed for the detection of HEV in serum and faeces on pig farms.[1 23] RT-PCR is a method with high specificity and sensitivity for detection of HEV RNA in serum and is frequently used to determine the presence of active HEV infection in individuals.[14 19 24 25] This methodology has certain drawbacks when used in screening and control programmes, including the need for qualified personnel to collect blood samples from animals, the associated animal stress and the economic cost. Moreover, the duration of viraemia in HEV-infected animals is generally short, lasting from one to two weeks postinfection, with more prolonged period of viral shedding in faeces of about three to eight weeks postinfection.[22 26] Collecting stools directly from the animals also has some drawbacks, including the need to handle the animals; however, the collection of stools from the pen floor does not cause any discomfort to animals. This sampling strategy is in increasing demand given the enhanced awareness on animal welfare and epidemiological surveillance of HEV in pigs.[16 27] Nevertheless, the effectiveness of this methodology compared with other invasive blood extraction methods routinely used has never been assessed.

The hypothesis was that the non-invasive screening approach could constitute a diagnostic tool at least as reliable as the invasive methods normally used to establish the HEV status of differently managed pig farms. Thus, the objective of this study was to evaluate the effectiveness of a non-invasive screening approach for determining the HEV status of pig farms under different management systems, based on random collection of stool samples from the pen floor followed by sequential real-time PCR analysis.

Materials and methods

Study design and sampling strategy

Sample collection of this prospective study took place between September and December 2015 on 26 pig farms in Andalusia, southern Spain (36°N–38°600 N, 1°750 W–7°250 W), a region with a high prevalence of HEV in people and pigs.[19 28 29] These farms were randomly selected, but represent the main pig production systems in Spain: (1) intensive (White and Iberian pigs), characterised by use of genetically improved breeds reared inside warehouses with commercial feed; and (2) extensive (Iberian pigs), with an important part of the breeding and fattening process occurring out in the countryside (in areas so-called 'dehesas'), feeding primarily on acorn and with more contact with other wild species.[19 30]

A total of 10 intensively managed farms of Large White/Landrace crosses, seven intensively managed farms of Iberian pigs and nine extensively managed farms of Iberian pigs, with populations of approximately 400 individuals each, were included and constituted the study population. The sample size was calculated assuming a farm-scale prevalence of 11 per cent, with a 95 per cent confidence level and an accepted error of 5 per cent. The number of pig farms sampled from each production system was proportional to the percentage of the different management systems in the census of farms in Andalusia. Within each management system, pig farms were randomly selected. Within each herd, 40 blood samples were obtained from 20 sows and 20 fattening pigs randomly selected from several batches, as well as 40 fresh and individual stool samples randomly collected from the floor of different batches and pens or yards in case of extensive systems (20 from sows and 20 from fattening pigs). The number of samples taken on each farm was chosen to ensure a 95 per cent probability of detecting at least one positive animal, assuming a minimum within-herd prevalence of 7 per cent.

The non-invasive screening approach to determine the HEV status of the pig farms was evaluated using the 40 stool samples from each farm, randomly numbered from 1 to 40. The samples were sequentially analysed by real-time PCR for HEV RNA, starting with stool sample number 1. If this sample was positive, the farm was considered 'HEV-positive' and no further testing for HEV in faeces was required. If the result was negative, stool samples numbered 2–40 were successively analysed one at a time until an HEV-positive sample was found. If none of the stool samples analysed was positive, the farm was considered 'HEV-negative'. The analysis was blinded with respect to the invasive method. This protocol is shown in figure 1.

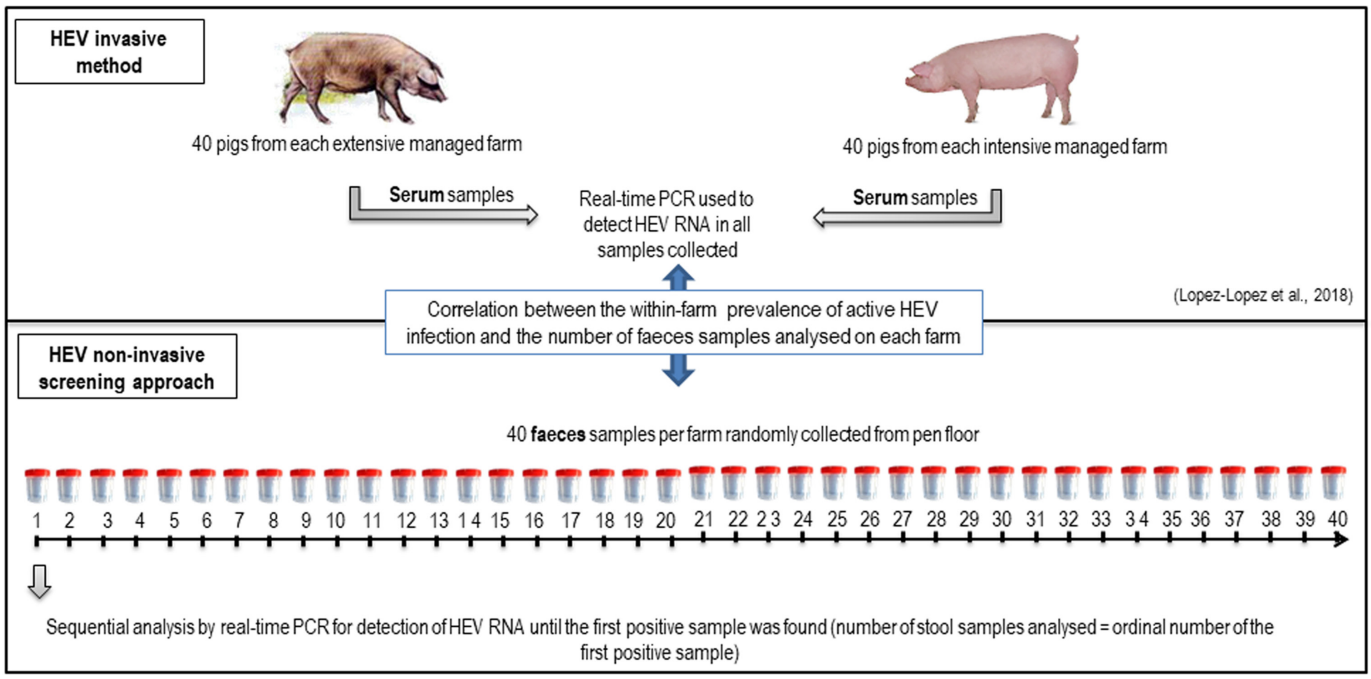

Figure 1 Schematic summary of the study design and sampling strategy on different management systems of pig farms (n=26). HEV, hepatitis E virus.

The invasive method used to confirm the HEV status and the within-farm prevalence of active HEV infection on each farm was detection of HEV RNA using real-time PCR in the 40 serum samples randomly chosen (figure 1). This sampling procedure can be found elsewhere.[19] A farm was considered HEV-positive by the invasive method when at least one animal was HEV-positive in serum, and HEV-negative when all the serum samples were HEV RNA-negative. The analysis was blinded with respect to the non-invasive screening approach.

RNA extraction and real-time PCR

All the samples (1040 blood and 1040 stool samples) were sent to the infectious diseases laboratory at the Maimonides Institute for Biomedical Research of Cordoba (IMIBIC), stored at 4°C and processed within 24 hours of collection. One gram of faeces and 200 µl of serum were stored at −80°C until nucleic acid extraction and analysis.

Extraction of viral RNA from 200 µl of faeces supernatant was carried out with the QIAamp Cador Pathogen Mini Kit (QIAgen, Hilden, Germany), using automated procedures (QIAcube, QIAgen). RNA was frozen at −80°C until analysis. For diagnosis of HEV infection, real-time PCR was performed using the LightCycler 480 (Roche, Basel, Switzerland), as described by Abravanel *et al.*[31] For the reaction, the QIAgen OneStep PCR Kit (QIAgen) was used with a sample concentration of 100 ng (RNA template) and 15 µMol of forward primer HEV5260 (5'-GGTGGTTTCTGGGGTGAC-3') and reverse primer HEV5330 (5'-AGGGGTTGGTTGGATGAA-3'). These primers bind to the ORF3 region of the virus (bases 5260 and 5330 in the standard viral chain), which is highly constant across genotypes and viral subtypes. The probe employed (20 µMol) was HEV5283 (5'-FAM-TGATTCTCAGCCCTTCGC-TAMRA-3'). The thermal profile was 50°C for 30 minutes, 95°C for 15 minutes, followed by 45 cycles of 94°C for 10 seconds, 55°C for 20 seconds and 72°C for 60 seconds. Any sample that had a cycle threshold value less than or equal to 35 was considered positive. The World Health Organization HEV standard strain supplied by the Paul Ehrlich Institute (code 6329/10) at a concentration of 250,000 iu/ml was used as positive control.

Blood samples were obtained without anticoagulant, processed and analysed as described by Lopez-Lopez *et al.*[19]

Biosafety aspects of research

Biological samples were treated as infectious material according to biosafety level 2, following the specific management, analysis and elimination measures indicated in the Waste and Contaminated Soils Act 22/2011 of 28th July of the Government of Spain.

Statistical analysis of data

The within-farm prevalence of active HEV infection on the farm was calculated by the invasive method using the ratio of positive samples in serum to the total number of samples analysed, with exact binomial confidence intervals of 95 per cent (95 per cent CI). The Shapiro-Wilk test was used to confirm non-normal distributions of data. Cohen's kappa coefficient was used to calculate intertest agreement between the two methods and thus compare the HEV status obtained with both. The relationship between the HEV invasive and non-invasive methods was studied by linear regression, using the non-parametric Spearman's rank-order correlation coefficient (Spearman's rho) to measure the association between within-farm prevalence of active HEV infection

Table 1 Within-farm prevalence of active HEV infection and first stool sample positive for virus on each farm

Farm	Management system	Pig breed	HEV-positive sera	Within-farm prevalence of active HEV infection by invasive method, % (95% confidence interval)	HEV status	First HEV-positive stool sample by non-invasive method
1	Intensive	Large White/Landrace	6/40	15 (7.1–29.1)	Positive	22
2	Intensive	Large White/Landrace	3/40	7.5 (2.6–19.9)	Positive	12
3	Intensive	Large White/Landrace	2/40	5 (1.4–16.5)	Positive	11
4	Intensive	Large White/Landrace	0/40	0 (0–8.8)	Negative	None
5	Intensive	Large White/Landrace	17/40	42.5 (28.5–57.8)	Positive	3
6	Intensive	Large White/Landrace	34/40	85 (70.9–92.9)	Positive	13
7	Intensive	Large White/Landrace	0/40	0 (0–8.8)	Negative	None
8	Intensive	Large White/Landrace	0/40	0 (0–8.8)	Negative	None
9	Intensive	Large White/Landrace	0/40	0 (0–8.8)	Negative	None
10	Intensive	Large White/Landrace	1/40	2.5 (0.4–12.9)	Positive	22
11	Intensive	Iberian	14/40	35 (22.1–50.5)	Positive	22
12	Intensive	Iberian	2/40	5 (1.4–16.5)	Positive	37
13	Intensive	Iberian	1/40	2.5 (0.4–12.9)	Positive	25
14	Intensive	Iberian	2/40	5 (1.4–16.5)	Positive	17
15	Intensive	Iberian	0/40	0 (0–8.8)	Negative	None
16	Intensive	Iberian	3/40	7.5 (2.6–19.9)	Positive	15
17	Intensive	Iberian	2/40	5 (1.4–16.5)	Positive	24
18	Extensive	Iberian	8/40	20 (10.5–34.8)	Positive	6
19	Extensive	Iberian	38/40	95 (83.5–98.6)	Positive	4
20	Extensive	Iberian	16/40	40 (26.3–55.4)	Positive	32
21	Extensive	Iberian	5/40	12.5 (5.5–26.1)	Positive	22
22	Extensive	Iberian	6/40	15 (7.1–29.1)	Positive	26
23	Extensive	Iberian	2/40	5 (1.4–16.5)	Positive	27
24	Extensive	Iberian	6/40	15 (7.1–29.1)	Positive	25
25	Extensive	Iberian	5/40	12.5 (5.5–26.1)	Positive	1
26	Extensive	Iberian	4/40	10 (4.0–23.0)	Positive	17

None means none of the stool samples was HEV-positive.
HEV, hepatitis E virus.

on each farm and the number of stool samples tested until the first HEV-positive sample was found. Analyses were done using the SPSS statistical software package (V.22.0; IBM, Somers, New York, USA), WinEpi software (V.11.36; Zaragoza University, Spain) and Epitools (Ausvet, Bruce, Australia).

Results

HEV RNA was detected by the non-invasive method in at least one faeces sample from 21 out of the 26 pig farms studied (80.8 per cent; 95 per cent CI: 62.1–91.5 per cent), which coincided exactly with those obtained by invasive methods in serum samples, with these farms being considered as HEV-positive. Regarding the management system, both invasive and non-invasive methods detected as HEV-positive 12 out of 17 intensive farms (70.6 per cent; 95 per cent CI: 46.9–86.7 per cent) and nine out of nine extensive farms (100 per cent; 95 per cent CI: 70.0–100 per cent). Likewise, the five farms (19.2 per cent; 95 per cent CI: 8.5–37.9 per cent) classified as HEV-negative using the non-invasive screening method were also considered HEV-negative in serum samples using the invasive method (table 1). Therefore, there is a very good intertest agreement (kappa value=1; 95 per cent CI: 1.0 to 1.0; se=0.196; P<0.00001) between the invasive and non-invasive methods, even on farms with a low within-farm prevalence of active HEV infection (ie, farms with a within-farm prevalence of 2.5 per cent, where HEV RNA was only detected in the serum of one animal out of 40 tested).

Table 1 shows the number of samples needed before one HEV-positive stool sample was detected. Interestingly, a negative statistical association was observed between the within-farm prevalence of active HEV infection in serum on each farm and the number of stool samples needed to be examined before an HEV-positive sample was found (Spearman's rho=−0.64; 95 per cent CI: −0.83 to −0.33; P=0.0004) (figure 2). This negative association was significantly higher in farms with intensive management system (Spearman's rho=−0.83; P<0.0001), but not in extensively managed pig farms (Spearman's rho=−0.09; P=0.81).

Discussion

The authors evaluated a non-invasive screening approach for determining the presence of HEV on intensively and extensively managed pig farms that required only random sampling of stools directly from the pen floor or yard, respectively. No false positives or negatives were detected when comparing the stool screening method with an invasive method in sera samples, regardless of the type of management system implemented. The agreement between the two

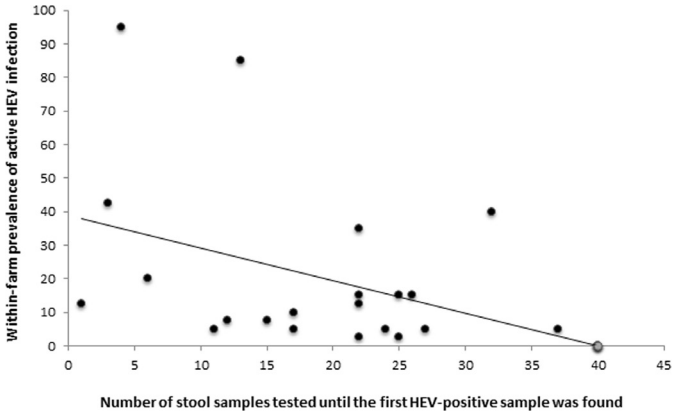

Figure 2 Correlation between invasive and non-invasive methods for detecting hepatitis E virus (HEV), comparing the within-farm prevalence of active HEV infection in each farm against the number of stool samples tested per farm before an HEV-positive sample was found. • HEV-positive farm; • HEV-negative farm.

methodologies for HEV diagnosis at the farm scale was 100 per cent in all the studied cases.

The screening method using faeces presents several advantages compared with the invasive blood extraction methodology: it is a non-invasive test that reduces discomfort, stress, pain and injury to the animal, and is also quick and easy to perform, since it can be carried out by staff with limited training and can be repeated without limitations. Moreover, this non-invasive method showed a high sensitivity for detection of HEV-positive farms, since pig farms with at least one animal HEV RNA-positive in serum also yielded one or more stool specimens positive to the virus, without the need to test every sample of faeces collected (n=40). The results suggest that this sampling strategy is an economic alternative to determining the HEV status of pig farms under different management systems.

In this sense, when the authors compared the within-farm prevalence of active HEV infection in serum and the number of stool samples examined before obtaining one HEV-positive, a significant negative relationship was found, which could present this non-invasive screening approach as an easy measure for obtaining valuable epidemiological information on the HEV status of farms. This signification is higher in intensive production systems, while it was absent in extensive production systems despite the optimum agreement between the two HEV diagnostic methods. This could be explained by pig population density being lower in outdoor systems than in confinement housing, and therefore the concentration of faeces is also lower and there is lower probability of obtaining a representative sample of the population with the same sampling size. Thus, in extensive production systems, the size of stool samples collected by farm could have been underestimated in relation to the number of the first HEV-positive stool found and to the within-farm prevalence of the farm, although this in turn has not affected the correct determination of herd-level status (HEV-positive or HEV-negative farm). Further studies collecting different stool sampling sizes in farms with extensive production management with smaller or larger pig populations are needed to better assess the potential epidemiological benefits of this non-invasive screening approach.

HEV RNA was detected in a higher percentage of extensively managed pig farms with respect to intensive ones by both methods analysed, suggesting a higher viral circulation in open systems.[18 19] This could be explained by the fact that HEV transmission and survival are favoured in the 'dehesas' system, where the interactions between HEV reservoirs, such as domestic animals (pigs) and wildlife (wild boar or deer), may occur.[29 32 33] These reservoirs could therefore be possible sources of HEV transmission.[34 35]

This study has several limitations that should be taken into account. First, not all serum samples of all pigs on negative farms were tested for HEV, which means that the possibility of false negative results cannot be completely ruled out. Secondly, the stool screening approach can be reliably applied with a sample size of 10 per cent of all the animals present on medium-sized pig farms, but the conclusions cannot be extrapolated to farms with larger pig populations as well as if the minimum prevalence is less than 7 per cent, where the number of samples needed should be increased. Thirdly, although HEV RNA in serum is frequently used to determine the presence of active infection in individuals, it would have been interesting to study the impact of HEV detection in pooled versus individual faecal samples in order to determine what would be the best representative pooled sample to determine the HEV status of pig farms, thus optimising or reducing economic costs.

In conclusion, the results of this study suggest that the non-invasive screening approach for HEV sequential detection in faeces from the floor can be an easy and economic method that can be reliably applied in a large-scale surveillance programme for determining the HEV status of pig farms under different management systems. Thus, the identification of HEV-positive farms could be the starting point for carrying out control measures to reduce the risk of HEV infection and transmission, both in farms or throughout the production network. Its applicability to wildlife species should be evaluated in future studies.

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Acknowledgements The authors thank the Agrarian and Fishery Management Agency (AGAPA) of the Government of Andalusia for providing samples and technical support, as well as Ismael Zafra for his technical support.

Funding This work was supported by the Ministry of Economy and Competitiveness (RD12/0017/0012) integrated in the National R+D+i Plan and cofinanced by the European Regional Development Fund and the Health Research Fund from the Institute of Health Carlos III (ISCIII) (PI16/01297), and the Spanish AIDS Research Network RD16/0025/0034-ISCIII-FEDER. AR-J is the recipient of a Miguel Servet Research Contract (CP18/00111) and MF is the recipient of a Sara Borrell Research Contract programme (CD18/00091), both from the Ministry of Science, Innovation, and Universities of Spain. JCG is supported by the FPU grant (FPU17/01319) of the Spanish Ministry of Education, Culture and Sport.

Competing interests None declared.

Ethics approval This study did not involve purposeful handling of animals, and the blood samples were not collected specifically for this study, taking advantage of samples obtained by government veterinarians for the surveillance programme of pigs of the Regional Government of Andalusia. Therefore, no ethical approval was deemed necessary for this study. Protocols, amendments and other resources were done according to the guidelines approved by the Regional Government of Andalusia following RD 53/2013 of the Ministry of Presidency of Spain, which establishes the rules for the protection of animals used in research.

Data availability statement Data are available upon reasonable request.

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