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Molecular and in vitro characterisation of hepatitis E virus from UK pigs

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ABSTRACT

Hepatitis E virus (HEV) infection is widespread in the global pig population. Although clinically inapparent in pigs, HEV infection is the cause of Hepatitis E in humans and transmission via the food chain has been established. Following a 2013 study that investigated prevalence of HEV infection in UK slaughter-age pigs samples indicating highest viral load were selected for further characterisation. High throughput sequencing was used to obtain the complete coding sequence from five samples. An in-frame insertion was observed within the HEV hypervariable region in two samples. To interrogate whether this mutation may be the cause of high-level viraemia and faecal shedding as observed in the sampled pigs virus isolation and culture was conducted. Based on viral growth kinetics there was no evidence that these insertions affected replication efficiency in vitro, suggesting as yet undetermined host factors may affect the course of infection and consequently the risk of foodborne transmission.

1. Introduction

Hepatitis E virus (HEV) is the cause of hepatitis E in humans. There are four HEV genotypes known to infect humans, HEV-1 to HEV-4. While genotypes HEV-1 and HEV-2 infect humans only, HEV-3 and HEV-4 have been detected in a number of animal species including pigs, wild-boar, deer and rabbit (Meng, 2013). There is a single report of human infection with Camelid HEV (HEV-7) (Lee et al., 2016).

Hepatitis E is typically an acute, self-limiting infection but with known risk groups. While in developing countries transmission via contaminated water supplies is key to the transmission cycle, autochthonous infections in Europe are presumed to predominantly involve the food chain (and to some extent blood transfusion) (EFSA, 2017). Since 2010 there has been an increase in the number of human cases within Europe (Adlhoch et al., 2016; ECDC, 2017). In the UK Public Health England introduced enhanced surveillance of HEV infections in England and Wales in 2003 and noted a continuous increase in autochthonous hepatitis E cases from 2010 to 2015, although most recent data shows a decrease in cases (PHE, 2016).

HEV is a small (27–34 nm diameter), non-enveloped, single-stranded, positive sense RNA virus of the family *Hepeviridae*. The linear genome is approximately 7.2 kb in length and comprises (5′–3′) a 7-methylguanylate cap structure, a short non-coding region (NCR), ORF1, ORF3, ORF2 (overlapping the majority of ORF3), a second NCR and a poly(A) tail. ORF1 encodes the non-structural polyprotein with the functional or putative domains of, from the N-terminus, methyltransferase, Y domain, papain-like cysteine protease, a hypervariable region (HVR), X or macro domain, helicase and RNA-dependant RNA polymerase (Koonin et al., 1992; Ahmad et al., 2011). The function of the HVR remains unclear, although findings from reverse genetics analyses suggest a role in viral replication (Pudupakam et al., 2011). ORF2 encodes the capsid protein while ORF3 encodes a small phosphoprotein whose function has still to be elucidated but is thought to be multifunctional with involvement in both replication and pathogenesis (Cao and Meng, 2012).

Pigs are a natural reservoir of HEV-3 and HEV-4 and infection is widespread in the pig population worldwide (Salines et al., 2017). Infection in pigs is clinically inapparent. Zoonotic transmission of HEV through the consumption of HEV-containing food, including pork products is established (EFSA, 2017; Pavio et al., 2015). Instigated by the increase in hepatitis E cases being recorded in England and Wales, the prevalence of HEV in UK slaughter-age pigs (n = 629) was investigated in 2013. Seroprevalence was 92.8% and 5.7% of pigs were viraemic (Grierson et al., 2015). Preliminary characterisation of circulating virus based on partial ORF2 sequences (304 nucleotides (nt); n = 23) demonstrated circulation of genotype HEV-3 in common with observations across Europe. Analyses of publically available HEV-3 nucleotide sequences indicate that there are currently three distinct clades or groupings of genotype 3 viruses. These grouping have not yet been formally classified but comprise of HEV-3 group 1 (G3-1; G3efg), HEV-3

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group 2 (G3-2; G3abchij) and a third group comprising of sequences obtained from rabbit samples and a single human case (G3ra) (Smith et al., 2015; Ijaz et al., 2014). The partial HEV-3 sequences characterised from the UK slaughter-age pigs in 2013 were predominantly HEV-3 group 1 (22 of 23), with a single HEV-3 group 2 sequence (Grierson et al., 2015).

Although there is growing interest in how HEV enters the food chain studies to date have mainly focused on surveillance and limited molecular characterisation. The study of HEV is restricted by the difficulty in isolation and culture, with viral heterogeneity often limiting complete molecular characterisation. Whilst the use of HTS may help, the use of faecal material as sample may cause hindrance due to the presence of contaminants. Here, the aim of this study was to further characterise HEV strains from UK pigs sampled in 2013 with a view to understanding transmission in to the food chain. Virus isolation and full genome analysis was conducted in order to better understand the circulating virus. In order to investigate genetic factors that may affect replication dynamics, viral growth kinetics were compared to genome sequences including investigation of the HVR as a potential factor influencing replication and hence the risk of HEV entering the food chain.

2. Material and methods

2.1. Samples

This work was a continuation of an analysis of caecal content samples that had been collected as part of the 2013 Zoonoses in UK Pigs Abattoir Study, a cross sectional study of pigs being slaughtered at 14 high-throughput abattoirs (Powell et al., 2016). At that time these samples (n = 629) along with paired plasma samples had been tested for detection of HEV RNA and antibody (Grierson et al., 2015). Five caecal samples were subsequently selected for analysis by high throughput sequencing (HTS) based on original test data (Ct value). Four samples (006, 022, 493 and 557) were selected based on lowest Ct values and a fifth sample (090) was selected to enable an assessment of the sensitivity of methodology (Table 1). These five samples had originated from different farms in Yorkshire (006, 022 and 090), Bedfordshire/ Hertfordshire (493) and East Riding/Lincolnshire (557). Samples had been collected from 4 different abattoirs; samples 006 and 557 had been collected from the same abattoir but in different months (January and April 2013 respectively).

2.2. Characterisation of the complete coding sequence of the HEV genome

For sample preparation a 1/10 suspension of caecal content was prepared in 50 mM Tris-HCl (pH 8), clarified, sequentially filtered through 0.45 µm and 0.20 µm filters and subsequently concentrated using an Amicon® Ultra centrifugal filter (100 K) (Millipore). Free nucleic acid was then digested using Omnicleave™ Endonuclease (Epicentre®). Two of the 5 samples analysed (006 and 022) had a relatively high concentration of virus and were prepared without concentration or digestion of free nucleic acid. RNA was subsequently extracted using TRIzol LS (Invitrogen) and the RNeasy Mini kit (Qiagen) following the protocol of Rasmussen et al. (2010).

Library preparation and sequencing using an Illumina MiSeq were performed as described by Marston et al. (2017). Genome sequence was obtained by iterative reference-guided mapping (initially using GenBank accession number JQ953665) using SeqMan NGen 11 (DNASTAR). Where reads did not provide complete coverage of coding sequence, where there was only single read coverage or where a review of the assembled reads identified areas of inconsistency in the pattern of assembly, sequence data encompassing these areas were obtained by conventional PCR using read-specific primers and Sanger sequencing.

2.3. Phylogenetic analysis

Full coding sequences were aligned using Clustal W (MegAlign™, DNASTAR). Phylogenetic analysis was performed using the neighbour-joining method in MEGA, v5 (bootstrap test of 1000 replicates) (Tamura et al., 2011). Reference sequences were taken from Smith et al. (2016).

2.4. Amplification of the HVR in ORF1

The HVR of genotypes HEV-1 to HEV-4 are surrounded by highly conserved amino acid sequences. All are bound at the C-terminus side by amino acid sequence starting RRLL (Purdy et al., 2012; Smith et al., 2012). Genotypes 3 and 4 are bound at the N-terminus side by sequence (T/V)SGFSS(D/C)FSP (Smith et al., 2012). A fragment of the HEV genome that encompassed the HVR was amplified by a nested conventional PCR using primer sets (Table 2) that had been designed from multiple sequence alignment of HEV-3 sequences (Bouquet et al. (2012) and study sequences). RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. Conventional PCR was performed using Dreamtaq Green

Table 1
Overview of test data and HTS analysis data for five caecal content samples.

ID	Detection of viral RNA (Grierson et al., 2015)		Overview of HTS analysis				Inoculum for investigation of replication	
	Caecal, Ct	Plasma, Ct	Sample preparation		Reads mapped (n)	Genome coverage relative to reference[a] (%)	Inoculum, Ct	Approx. copy number[b]
			Ct	ng/µl (elute #)				
006	24.23	23.7	25.32	6.7 (3)	2156d[d] 3370	99.5 99.8[f]	24.20	10^6
022	24.71	25.5	25.41	9.2 (3)	9005[e]	100[f]	23.77	10^6
493	32.78	ND	26.21	**3.0 (1)**	5297[d]	98	31.86	10^3
557	33.04[c]	34.7	29.59	2.8 (1)	169[d]	83	29.37	10^4
090	36.26	ND	32.96	1.8 (2)	44[e]	31	–	–

Ct, cycle threshold; ND, not detected.
[a] JQ953665
[b] 200ul volume used to inoculate in 6-well plate format
[c] Repeat test data (Ct 28.93) indicated inhibition in original PCR test data
[d] ½ MiSeq slide coverage
[e] full MiSeq slide coverage
[f] Genome subsequently shown to contain insert in HVR

Table 2
Primer sequences for amplification of HVR.

Primer set	Primer	Nucleotide sequence (5′−3′)
First round	HVR_2125_F	TTTAYACNCGDACYTGGTC
	HVR_2468_R	CAGCCARTCACARTCHGACTC
Nested	HVR_2133_F1[1]	CGGACYTGGTCYACATCHGG
	HVR_2440_R1	GAGCCYGCRTAVACCTTRGC

[1] Encompasses first 8 nt of HVR.

Mastermix (Thermo Fisher Scientific) and with primers at a final concentration of 1 μM. PCR product was visualised by gel electrophoresis.

2.5. Investigation of the replication of HEV

Virus isolation was carried out using PLC/PRF/5 cells (original source: ATCC® CRL-8024™) in 2D culture. Inoculation and propagation in a 6-well plate format was performed as described by Takahashi et al. (2010) except that 50% of the culture medium was replaced with fresh medium every 2 or 3 days and for monitoring of viral propagation an aliquot of the removed media (140 μl) was added to AVL buffer (Qiagen) and stored at −20 °C until extraction. Nucleic acid was extracted using the QIAamp Viral RNA mini kit (Qiagen) and HEV RNA detected using a TaqMan real-time RT-PCR (Garson et al., 2012). Quantitative data where presented was extrapolated from titration of the WHO International Standard (PEI code 6329/10, Paul-Ehrlich-Institut, Germany) that had been diluted in a HEV RNA negative caecal content sample. A PCR positive control was included to monitor intra-assay reproducibility (range Ct 35.57–36.77).

Firstly, the 2D cell culture protocol was established in the laboratory using material known to contain viable virus, a HEV RNA positive cell culture supernatant from an earlier study (Berto et al., 2013). The supernatant was inoculated neat (~10^6 copies per well; 4 replicates) and at a 1/10 dilution in PBS (2 replicates). A negative control (PBS inoculum) was included. Subsequently, the replication of HEV in samples 006, 022, 493 and 557 was investigated. Inoculum was prepared by diluting caecal contents 1/10 in DMEM and homogenised samples were then clarified and sequentially filtered through 0.45 μm and 0.20 μm filters. Propagation of HEV in vitro appears to be dose dependant (minimal HEV load) (Okamoto, 2013) and therefore isolation of sample 090 was not attempted. Viral copy number in the inoculums was determined. A HEV RNA negative caecal content sample was processed in parallel as a negative control. The cell culture supernatant used initially to establish the protocol was used as a positive control. All samples were tested in duplicate.

3. Results

3.1. Characterisation of the complete coding sequence of the HEV genome

Five HEV RNA positive caecal content samples were sequenced. Reference-guided mapping of reads provided 31–100% coverage of the reference sequence (Table 1). An inconsistent pattern of read assembly within ORF1 was identified for sample 022 (isolated region of single read coverage) and subsequent sequence validation by PCR using read-specific primers identified an 84nt in-frame insertion within the HVR of ORF1 for sample 022. Review of the read assembly for sample 006 identified two distinct patterns of nucleotide sequence in the assembly, clearly indicating the presence of a second strain. Read-specific primers were designed on areas of mismatch both upstream and downstream of the target region of primers of Jothikumar et al. (2006) and used to obtain longer strain-specific sequence from which to assemble two genome sequences (006_1 and 006_2). An in-frame insertion of 81 bp was identified within the ORF 1 HVR of 006_2. The coding sequence of the two genomes characterised from sample 006 shared 87.6% identity, with 86.2% nucleotide identity in ORF1, 90.5% in ORF2 and 95.9% in ORF3.

The insertions identified in sample 006 and 022 appear to be derived from an insertion of duplicated viral sequences from immediately upstream of the insertion (Fig. 1).

Complete genome sequence was obtained from sample 022 (UK-SHEV-022, 7323 nt; GenBank accession number MH184581), while two complete coding sequences were obtained from sample 006 (UK-SHEV-006_1, 7132 nt, MH184579 and UK-SHEV-006_2, 7213 nt, MH184580). Incomplete sequences from samples 557 (1 gap of 170 nt), 493 (17 gaps of 3–284 nt; areas of single read coverage) and 090 (44 reads) were used to design read-specific primers for gap filling by PCR and Sanger sequencing. Complete coding sequence was subsequently obtained from all 5 samples (UK-SHEV-090, 7132 nt, MH184582, UK-SHEV-493, 7132 nt, MH184583 and UK-SHEV-557, 7132 nt, MH184584).

The 6 HEV sequences from UK pigs shared 85.9–98.5% nucleotide identity in ORF1, 88.3–98.2% identity in ORF2 and 94.9–100% identity in ORF3. Sequences 006_1 and 557 shared highest similarity (98.5% in ORF1, 98.2% in ORF2 and 100% in ORF3).

All 6 sequences were genotype HEV-3 and clustered within group 1 (subtype 3e) (Fig. 2), consistent with earlier findings from the partial characterisation of ORF2 from these samples (Grierson et al., 2015).

3.2. Amplification of the HVR in ORF1

HTS identified insertions in the HVR in two of the six characterised HEV genomes. A nested conventional PCR was subsequently used to investigate the presence of insertions within the HVR of HEV. The size of the PCR product visualised for samples 006 and 022 were consistent with the genome information obtained from full genome sequencing (HVR insertions present in both samples; dual HEV infection in 006, one with and one without HVR insertion) as well as the absence of insertions in samples 557, 493 and 090 (data not shown). Further caecal content samples that had been collected in 2013 were investigated (n = 20) and PCR product was visible for 13 samples and indicated that the HVR in these samples were all of the expected size (308nt) and did not contain insertions (data not shown; original test data Ct 33.7–37.6). No product was visible for 7 samples (original test data Ct 36.4–37.5).

3.3. Investigation of the replication of HEV

Firstly, the 2D cell culture protocol for propagation of HEV (Takahashi et al., 2010) was established. Cell cultures were inoculated when cells reached confluence but in the days following inoculation it was observed that the cell monolayers were not maintained and 3-dimensional aggregates appeared to develop. This was also observed in subsequent experiments. Time-point sampling and testing of the cell culture supernatants from 2 to 36 days post infection (dpi) demonstrated reproducible propagation of the virus (Fig. 3). Propagation of virus in the 1/10 diluted inoculum was delayed relative to the undiluted inoculum and commenced from ~20 dpi. HEV RNA was not detected at any time-point in the negative control (PBS inoculum).

Study samples were subsequently investigated (Table 1), along with

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006_1   TSGFSSDFSPPEAAFAAPAPDGGLPLGTPPSVSDVWVLPPPSEGSVA----------ALLPAAPVSKPASPPSXTTPRPPVRKLPSPPPARTRRLL
006_2   TSGFSSDFSPPEAALAAPAFDGGLPPGTPSSTSGVWVFPPAPEGSVATPSLAAPA-KPAGLPSPAFPPLLEGSETAPPLAAPVSKPTDLSSPTI SKPPVRKPPSPPPVRTRRLL
022     TSGFSSDFSPPEAAFAAPAPGGVFPPGTPSSASDVWVLPPPSEGSVAAPPLAAPASRPVSLPSLALPPPSEGSVTAPPLAAPASKPTGPPGPAPSKPPARKPPPPPPARTRRLL
090     TSGFSSDFSPPEATFAAPAPDGGLPPGTPSFASDVWVLPPPSEGSVV----------ALPPAAPASMSTSPPGPVTPRFPVRKPPSPPPARTRRLL
493     TSGFSSDFSPPEAAFAAPASCGGLPPGTPSSVSDVWVLPPPSEGSVV----------APPPTAPVSKPTSPPGPAI PRPPVCKPSSPPPARTRRLL
557     TSGFSSNFSPPEAAFAAPAPDGGLPLGTPSSVSDVWVLPPPSEGSVA----------ASLPAAPVSKPASPPSPATPRPPVRKLPSPPPARTRRLL
```

Fig. 1. Predicted amino acid sequence of the HVR of HEV genomes. Apparent duplicated viral sequence is marked (box).

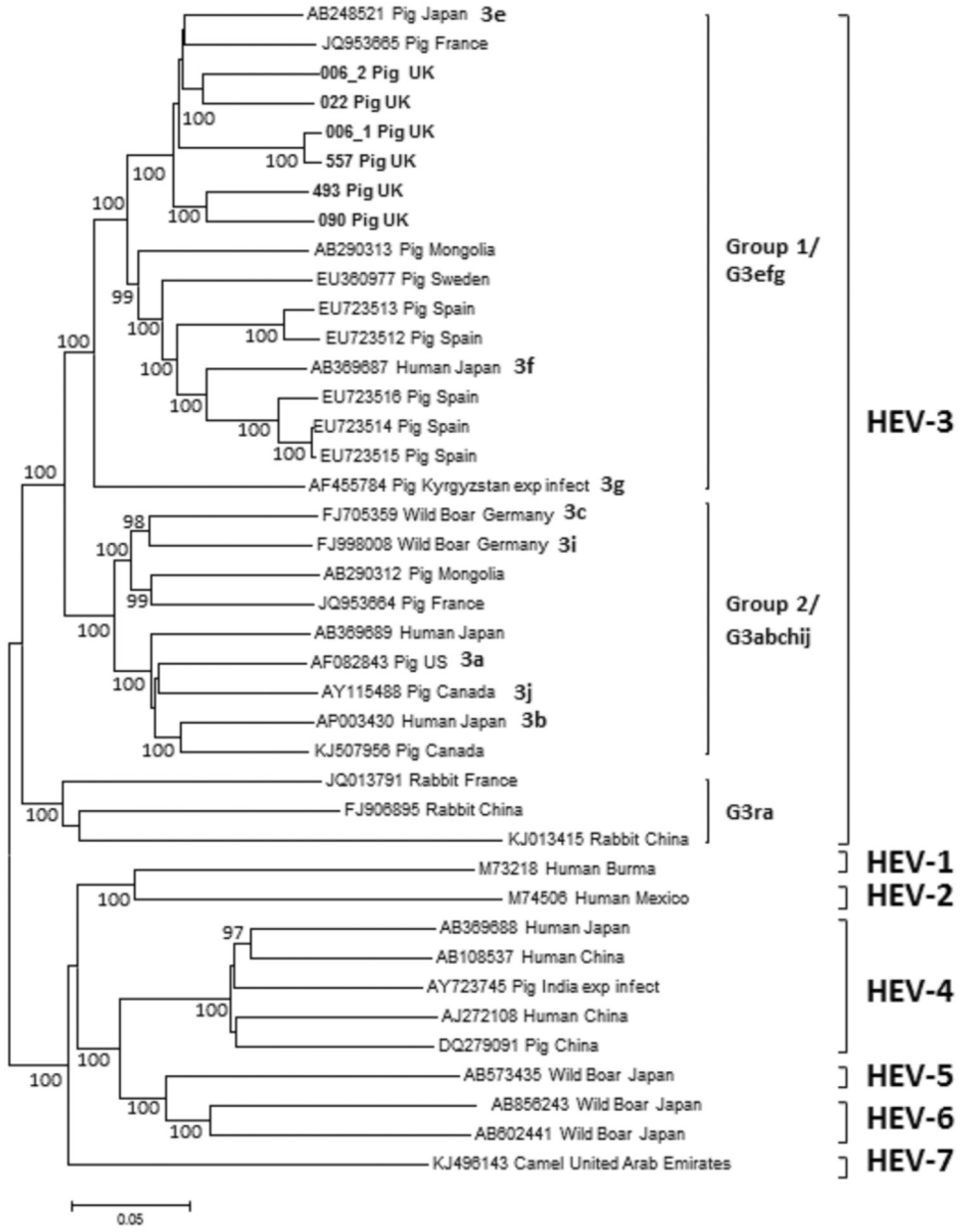

Fig. 2. Phylogenetic dendrogram depicting genetic distance based on the complete coding sequence of hepatitis E virus. Sequences were aligned using MegAlign™ (DNASTAR) and genetic distances calculated using MEGA5 (Neighbour-joining). Analysis includes 34 reference genotypes and HEV-3 subtypes (Smith et al., 2016). Bootstrap values > 70% are presented. exp infect, experimentally infected.

a positive control inoculum (Ct 22.46; approximately 10^6 copies). Cytopathic effect was observed within 4 dpi in both wells for sample 493. Investigation later confirmed the presence of a porcine sapelovirus in this sample (data not shown). Time-point sampling continued for all other samples up to 38 dpi. Propagation profiles for samples 006 and 022 were similar and from ~22 dpi paralleled the replication rate of the positive control (Fig. 4). The HVR insertion was still present in HEV RNA extracted from both samples at 38 dpi, although of the two strains present in sample 006 the strain without the insertion was selected for during the period of culture, as evidenced by visualisation of product by gel electrophoresis: PCR products were visible at approximately equally strong intensity in the original samples but product from the strain with the insertion was only weakly visible at 38 dpi (data not shown). HEV RNA in cell culture supernatant for sample 557 was sporadically detected in one or the other replicate until 17 dpi, from which point the propagation rate paralleled that of samples 006 and 022 and the positive control (Fig. 4).

4. Discussion

In order to better understand the biology of HEV circulating in UK pigs and potentially identify factors that may be involved in transmission of it to the pork food chain, full genome sequencing was carried out on archived samples with the highest viral load i.e. those that proposed the greatest risk regarding entry to the food chain. Complete coding sequences of six HEV genomes from five infected UK pigs were characterised. An in-frame insertion in the HEV HVR was observed in two infected pigs. One of the two HEV strains characterised from sample 006 (006_2) included an 81 nt insertion while that from sample 022 included an 84 nt insertion. Interestingly, these insertions were found in the two samples with the highest viral load in plasma and caecal samples in 2013 study (Table 1), indicating a possible role of insertions into the HVR for replication efficiency.

A duplication of viral sequence into the HVR has been reported previously with the presence of an 87 nt insertion in the HEV HVR from

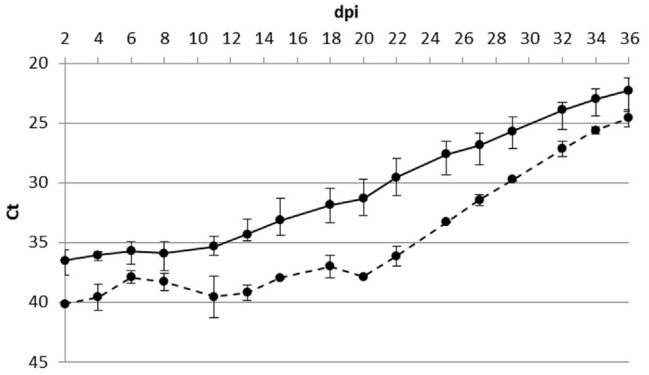

Fig. 3. Establishment of the 2D cell culture protocol of Takahashi et al. (2010) for reproducible propagation of HEV (using an irrelevant inoculum). HEV RNA was detected in cell culture supernatants from time-point sampling of propagation in PLC/PRF/5 cells. Four replicates of undiluted inoculum (solid line) and two replicates of a 1/10 dilution (dashed line) were tested. Range bars are indicated (lowest and highest value). dpi, days post-infection, Ct, cycle threshold.

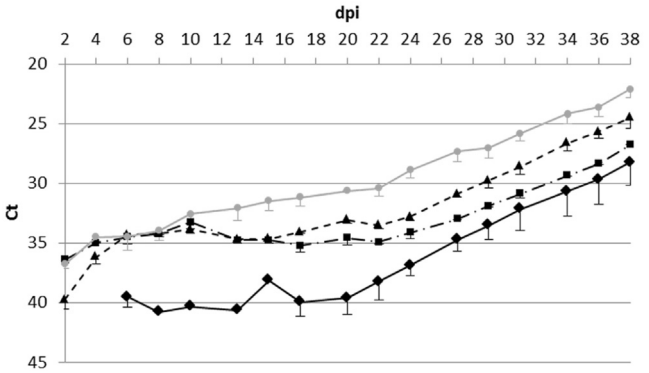

Fig. 4. Detection of HEV RNA in cell culture supernatants from time-point sampling of propagation in PLC/PRF/5 cells. Two replicates of positive control (–●–), of sample 006 (–▲–), of sample 022 (–■–) and of sample 557 (-◆-) were tested. Upper range bars are indicated (highest value). dpi, days post-infection, Ct, cycle threshold.

three pigs sampled from the same farm in Spain (Peralta et al., 2009) and subsequently reported to be the result of sequence duplication in an adjacent region of the HVR (Purdy et al., 2012). This appears to also be the case for the current findings (Fig. 1). The finding of insertions in the HEV HVR has also been characterised from human infections, including insertions reported to be as a result of sequence duplication (Purdy et al., 2012; Legrand-Abravanel et al., 2009; Johne et al., 2014) or host-derived insertions (Shukla et al., 2011; Nguyen et al., 2012).

It has been suggested that natural insertions in the HVR enhance viral replication. A natural insertion in the HVR was associated with efficient replication in cell culture, relative to three other strains (Johne et al., 2014). Genomes with host-derived insertions in the HVR existing as low frequency/ minor variants in faecal samples were selected for during attempts to propagate HEV strains (Shukla et al., 2011; Nguyen et al., 2012). The reason for this apparent replication advantage in vitro is not known. In our study it was hypothesised that the HEV genomes with the virus-derived insertions would replicate more efficiently in vitro relative to virus without insertions in the HVR.

The function of the HVR remains unclear, a potential role in viral replication has still to be elucidated but data from reverse genetic analyses suggest that the complete naturally-occurring HVR sequence is not needed for viral replication in vitro (Pudupakam et al., 2009, 2011). However, deletions within the HVR result in reductions in replication efficiency, with greater impact when located at the N-terminal or central region and where larger deletions led to a greater reduction in efficiency in vitro (Pudupakam et al., 2011).

Here, over the course of culture for 38 days there was no obvious evidence of enhanced replication properties for samples 006 and 022 (Fig. 4). Indeed, of the two strains present in sample 006 the strain without the insertion was selected for during the period of culture. The reason for this apparent discrepancy may be manifold. The enhanced replication efficiencies that have been reported were demonstrated for human HEV in vitro with host (human)-derived insertions using human cell lines (Johne et al., 2014; Shukla et al., 2011; Nguyen et al., 2012). In this study replication had been investigated using pig viruses with virus-derived insertions and the human cell line PLC/PRF/5 (Takahashi et al., 2010; Berto et al., 2013). Although there was no evidence in this study of enhanced replication efficiency in vitro, host factors were not investigated and there is evidence that the HVR has a role to play in the course of infection in the host. *In vivo*, near-complete deletion of the HVR was found to affect infection dynamics with delayed seroconversion and absence of faecal shedding (up to 10 weeks p.i) in pigs (Pudupakam et al., 2009). Hence follow on in vivo studies comparing HEV strains with or without the HVR insertion are needed to confirm the effect of the observed HVR mutations.

With the increase in autochonous human HEV cases across Europe the spotlight is on potential sources of infection with the greatest suspicion, backed by epidemiological studies, being levied towards pigs and the pork food chain. There are numerous studies highlighting the ubiquitous nature of HEV infections in pigs worldwide however lack of interest (with HEV being asymptomatic in pigs) and lack of tools have hindered investigations. Despite practical (laboratory) challenges here viral isolates and full genome sequences were obtained from field samples which enabled the investigation of HEV genetic variation on viral growth kinetics. However, as demonstrated above, to isolate HEV from field samples is not without its problems. As HEV is transmitted via the faecal-oral route and as such sampling is not invasive faeces is the sample of choice for many pig HEV studies. As seen here use of a similar sample type led to the contamination of cell culture with the detection of a coinfection with porcine sapelovirus (cytopathic) in sample 493. The use of caecal contents for HTS also has its issues with the low level of virus in comparison to the level of contaminants and host material. As can be seen in Table 1 an increase in 3 Cts results in approximately 50% drop in genome coverage. Here, the focus was on those samples with the highest viral loads in relation to risk of entry to the food chain but also for practical reasons as described above. In order to gain a clear overall perspective of HEV on farm dynamics methods that can allow characterisation of a range of viral load samples are needed. As seen from HVR data reported here further work is needed in order to better understand the factors that may affect replication in the pig and those that may influence HEV entering the food chain.

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