Feasibility study for in vitro analysis of infectious foodborne HEV.

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1. Project summary

Hepatitis E virus (HEV) is a member of the Hepeviridae family capable of infecting humans producing a range of symptoms from mild disease to kidney failure. Epidemiological evidence suggests that hepatitis E genotype III and IV cases may be associated with the consumption of undercooked pork meat, offal and processed products such as sausages [1]. A study carried out

by the Animal Health and Veterinary Laboratories Agency (AHVLA), found hepatitis E virus contamination in the UK pork production chain and that 10% of a small sample of retail pork sausages were contaminated with the virus [2]. Furthermore, studies have confirmed the presence of HEV in the food chain and the foodborne transmission of Hepatitis E virus to humans [reviewed in 5]. Likewise, Scottish shellfish at retail [6] have also been found positive for HEV viral nucleic acid and some preliminary studies indicate that the virus is also detectable in soft fruits (L Scobie; unpublished data).

There are current misunderstandings in what this data represents, and these studies have raised further questions concerning the infectivity of the virus, the processing of these foods by industry and the cooking and/or preparation by caterers and consumers.

There are significant gaps in the knowledge around viral infectivity, in particular the nature of the preparation of food matrices to isolate the virus, and also with respect to a consistent and suitable assay for confirming infectivity [1,3]. Currently, there is no suitable test for infectivity, and, in addition, we have no knowledge if specific food items would be detrimental to cells when assessing the presence of infectious virus *in vitro*.

The FSA finalised a comprehensive critical review on the approaches to assess the infectivity of the HEV virus which is published [3] recommending that a cell culture based method should be developed for use with food. In order to proceed with the development of an infectivity culture method, there is a requirement to assess if food matrices are detrimental to cell culture cell survival. Other issues that may have affected the ability to develop a consistent method are the length of time the virally contaminated sample is exposed to the cells and the concentration of the virus present. In most cases, the sample is only exposed to the cells for around 1 hour and it has been shown that if the concentration is less that $1x10^3$ copies then infection is not established [3,5,10,11].

2. Project aims and research deliverables

The objective of this work was to determine the effect of relevant food homogenate on cells that may be used as culture infectivity assays, taking into account previously published studies [7,8.9]. Cultured cells can be deleteriously affected by substances co-extracted from pork products with HEV during sample treatment (Johne, unpublished), and only three studies have reported the successful isolation of HEV from pork products by cell culture [7,8,9] The aim of this study, is not to confirm if the foods selected contain HEV or indeed, infect the chosen cells, but to simply assess the effect of the matrix itself on cell survival over time and at varying dilutions. The findings will contribute to knowledge gaps in terms of defining if the development of an *in vitro* infectivity assay for foodborne infection is indeed feasible. This will generate the evidence in this area to begin to address both industry and consumer concerns and provide more relevant assays to assess food safety.

3. Methods

3.1 Preparation of food homogenate

In order to determine if food homogenates are cytotoxic to target cells, selected food matrices, at the direction of the FSA, including high and low value pork sausage (due to variations in meat content), pate, shellfish, soft fruits, leafy vegetables and pork chops were tested for their toxicity *in vitro*. Weights and volumes were scaled down to accommodate the large number of experiments required. Composition of the food stuffs is presented in Appendix 1: Summary table of the food homogenates composition. Food homogenates (FH) were prepared from: strawberry (st), raspberry (Ras), lettuce (L), pork sausages with 40% meat (PS1), pork sausages with 97% meat (PS2), British pork chops (PC), smooth brussels paté with madeira jelly (SBP), cooked

mussels (CM). Finally, raw mussels (RM) and raw oysters (RO) were purchased fresh.

In brief, 30 mg (±3 mg) sections composed of the entire food stuff without any prior treatment were transferred into a 1.5 ml screw cap Eppendorf containing sterile 1.0 mm glass beads. 600 µl of sterile phosphate buffered saline (PBS pH7.4, Merck, Dorset, UK) was added to each section. Then samples were homogenised at 50Hz for 2.5 minutes using the TissueLyser LT (Qiagen, Surrey, UK). After homogenisation, samples were centrifuged at 10,000xg for 20mins at 4°C. 500µl of supernatant was transferred to a fresh 1.5 ml Eppendorf tube after a 0.45uM (Sarstedt, Leicester, UK) filtration step and stored in aliquots at -20°C. This method was selected for two reasons; 1) our recent studies to determine the optimal method for extraction of HEV from food as per the FSA study FS307033 based on previous studies [12-14] and 2) previous literature where food was suspended in PBS for infectivity studies [7,8,9]. The study by Takahashi [7] and Berto et al [9] used suspensions in PBS; the Berto study [8] used a larger volume and resuspended in culture medium. Berto et al [9] also diluted the sample in media at 1:5 as we have done here.

3.2 Maintenance of cells in culture and assay for toxicity

To determine if the homogenate contains inhibitors or other content that is detrimental to the cells, the homogenates were added to selected cell lines that have been utilised to test infectivity of HEV *in vitro* [3]. Cells were purchased from ATCC (American Type Culture Collection): A549 (ATCC cell line number CCL-185), HepG2/C3A (ATCC cell line number HB-8065) and PLC/PFR/5 (ATCC cell line number CRL-8024). Cells were grown in 15ml Gibco Dulbecco's modified Eagle's medium: Nutrient Mixture F-12, DMEM: F12 medium (ThermoFisher Scientific, Glasgow, UK) supplemented with 10% Gibco heat-inactivated Foetal bovine serum (FBS) (ThermoFisher Scientific) and 1% penicillin/Streptomycin 100X (Gibco, Fisher Scientific, 5,000U/ml) and 1% L-Glutamine (ThermoFisher Scientific) in T75 flasks (Corning, Dorset, UK). and maintained in culture at a density between 1- 3 x 105 cells/ml and sub-cultured every 5 days, until 70-80% confluency. Sub-culturing of cells was performed by trypsinisation with 2ml of trypsin-EDTA 10X (0.5%, no phenol red (Gibco) and incubated for 5-10 minutes at 37°C (5 min for A549 cells, 7 min for HepG2 cells and 10 min for PLC cells). 8ml of DMEM:F12 was added to the cells to neutralise the trypsin activity. The cells were centrifuged at 200xg for 5 minutes. Cell imaging was taken using an EVOS microscope.

To assess toxicity of the FH, a MTT assay (Invitrogen™ CyQUANT™ MTT Cell Viability Assay) was purchased and used to assess cell survival *in vitro*. In brief, A549, HepG2/C3A and PLC/PFR/5 cells were seeded in 96 well plates at a density of $1x10^4$ cells/well and cells were incubated at 37°C with 5% CO_2 for 24h. Media was removed from the cells and the FH were added to the wells as neat in PBS, 1:2 dilution and 1:5 dilution PBS:media concentration (stock concentration of the FH is 50mg/ml) then incubated at 37°C with 5% CO_2 for 24, 48 and 72 hours.

At each duplicate time point, the supernatant was removed and 10µl of MTT solution and 80µl 80 µl of cell culture medium were added to each well. Plates were then incubated at 37°C for 3 hours. After incubation, 100µl of DMSO was added to each well with pipetting to fully dissolve the MTT formazan. Plates were further incubated at room temperature in the dark for 10 minutes. Absorbance was read at OD=590nm using Epoque plate reader. All experiments were repeated in duplicate. PBS, dilutions with PBS and media and media only were used as controls for the effect on the cells.

3.3 Statistical analysis

Results are expressed as Mean ±SEM. Statically significant differences were determined using PRISM version 4 Software by a Two-Way ANOVA Bonferroni Post-test with $P<0.001$ as significant.

4. Results

The 11 homogenates were tested on A549, HepG2/C3A and PLC/PFR/5 cells as neat, 1:2 and 1:5 dilution concentrations and the toxicity of these homogenates, as measured by cell death were tested after 24, 48 and 72 hours. Figure 1 illustrates the cell morphology of the aforementioned cell lines prior to treatment at 72h post addition of FH.

Following culture of the cells, each cell type was treated with the FH in duplicate. Figure 2 shows the cell morphology in A549 cells at 72h post incubation at a 1:2 dilution. In panel 1, the control shows clear growth of the cells, however, panels 5 (PS1), 6 (PS2), 8 (SBP), 10 (water), 11 (RM) and 12 (RO) show a cytopathic effect (CPE) on the A549 cells by exhibiting rounding up and clumping of the cells compared to the control.

To confirm this CPE and subsequent evidence of cell death, an MTT assay was used at time 0, 24, 48 and 72 hours post treatment.

Figure 1. Untreated (A) A549, (B) PLC/PFR/5 and (C) HepG2/C3A cells after 24h following seeding in 96 well plate at 1x10^4 cells/well in DMEM:F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO_2 (x10).

A

B

C

Figure 2. Viability and morphology of A549 cells after 72h following addition of 1:2 dilution of various homogenates samples to the cells. Panel 1 is untreated cells, panels 2-11 are the FH. Cells were seeded for 24h in 96 well plate at 1x104 cells/well in DMEM:F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO_2 (x10).

Figure 3. Cytotoxic effect of the food homogenates on A549 cells viability by MTT assay test. After 24h of cell seeding in a 96-well plate, homogenates were added to the cells and incubated at 37°C for 24h (A), 48h (B) and 72h (C). Values are presented as percentage of cell death (±SEM) of n=2. The data were analysed by two-Way ANOVA test, *P<0.05, **P<0.01 and ***P<0.001 versus relevant control (untreated cells in cell media only).

A

B

C

Figure 3 shows the percentage of cell death after 24 hours (Figure 3A), 48 hours (Figure 3B) and 72 hours (Figure 3C) for all dilutions. In summary, for A549 cells, homogenates from pate (SBP) and raw oysters (RO) were detrimental to cells regardless of whether they were in PBS or diluted with media showing greater than 35% cell death ($p<0.001$) in comparison to untreated controls. For the other food homogenates, as expected, cell death was reduced when they were diluted out with media at 24 hours (Figure 3A, B and C), however, an overall detrimental effect was still observed. Interestingly, as the time in contact was increased to 48 hours, raw mussels (RM) contributed more to cell death as did FH from strawberries (St) and Pork sausage (PS1) at 72 hours, suggesting that the longer contact time with the FH led to a more detrimental effect on the cells in culture.

For the neat PBS control, again as expected, when added to cells alone, it was observed that after 24, 48 and 72 hours we saw 22%, 52% and 52% cell death respectively. When diluted out with media we saw an average percentage cell death of 25% at a 1:2 dilution and 22% at a 1:5 dilution over all time points.

The analysis was then repeated using PLC/PRF/5 and HepG2 cells.

In figure 4, CPE is observed for almost all of the FH at a 1:2 dilution treatment. MTT analysis is shown in figure 5 for PLC/PRF/5 cells; we observed that all neat FH, except PC were exhibiting >60% death after 24 hours (Figure 5A). At 48 hours (Figure 5B), results were similar and after 72h, all FH treatment showed cell death >90% (Figure 5C). Dilution of the FH at 1:2 and 1:5 showed that cell death was reduced compared to neat (Figure 5A) but cell survival at 48 hours (figure 5B) and 72hours (Figure 5C) showed increasing significant levels of cell death.

Overall, pate had the most significant effect on the cells at the 24hour time point (46% cell death) and by 72 hours all FH affected the cells at a significant level regardless of dilution factor (Figure 5C). This was in contrast to the PBS only control which showed only an average 6.5% cell death at 1:5 and 19% at 1:2 dilution suggesting that these cells may be more sensitive to any FH content that would affect the cells.
Finally, for HepG2 cells, as seen in the A549 cells, a CPE was seen with the differing FHs (Figure 6). Again, like in the A549 cells, pate and RO showed increased cell death at all dilutions and time points (Figure 7). Again, it was observed that deleterious effects on the cells were observed also for ST, PS1 and RM at 72 hours (Figure 7C). This was in contrast to the PBS only control which showed only an average 7% cell death at 1:5 and 25% at a 1:2 dilution.

Figure 4. Viability and morphology of PLC/PRF/5 cells after 72h following addition of 1:2 dilution of various homogenates samples to the cells. Panel 1 is untreated cells, panels 2-11 are the FH. Cells were seeded for 24h in 96 well plate at 1x10^4 cells/well in DMEM:F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO_2 (x10).

Figure 5. Cytotoxic effect of the food homogenates on PLC/PRF/5 cells viability by MTT assay test. After 24h of cell seeding in a 96-well plate, homogenates were added to the

cells and incubated at 37°C for 24h (A), 48h (B) and 72h (C). Then MTT assay was followed. Values are presented as percentage of death (±SEM) of n=2. The data were analysed by two-Way ANOVA test, *P<0.05, **P<0.01 and ***P<0.001 versus relevant control (untreated cells in cell media only).

A

B

C

Figure 6. Images depicting the viability of HepG2 cells after 72h following addition of 1:2 dilution of various homogenates samples to the cells. Panel 1 is untreated cells, panels 2-11 are the FH. Cells were seeded for 24h in 96 well plate at 1x10^4 cells/well in DMEM:F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO_2 (x10).

Figure 7. Cytotoxic effect of the food homogenates on HepG2 cells viability by MTT assay test. After 24h of cell seeding in a 96-well plate, homogenates were added to the cells and incubated at 37°C for 24h (A), 48h (B) and 72h (C). Then MTT assay was followed. Values are presented as percentage of death (±SEM) of n=2. The data were analysed by two-Way ANOVA test, *P<0.05, **P<0.01 and ***P<0.001 versus relevant control (untreated cells in cell media only).

A

B

C

5. Summary

The cytotoxic effect of the FH was tested via the viability of the three cell lines to tolerate varied concentrations of the FH as well as over time. Cell death of >35% (cell viability less than 65% of the control untreated cells with DMEM only) is considered significant.

It is apparent from this study, that varying FH have differing effects on cells *in vitro*. The PBS control, although not containing any supplementary media, was able to support cell survival for a period of 72 hours in the A549 cells but not in the others. When diluted with media, as expected, cell survival was promoted in all cell lines evaluated. Interestingly, only two FH were particularly toxic to all cells at 48 hours; pate and raw oyster homogenate. At 72 hours, effects from other FH were observed, for example, St, PS1 and RM.

In regard to the target cell lines, PLC/PRF/5 cells appeared to be more sensitive than the HepG2 or A549. The most robust cell line over a time period for testing the presence of HEV infectivity appears to be the A549 cell line which should be the starting point. A549 cells were duplicating faster in wells which may contribute to their being more tolerant. After initial investigation of the FH on target cells, it would follow that spiking of FH with HEV virus at differing concentrations is the next step to confirm if it is possible to then subsequently infect cells in these matrices. This is important as FH are expected to have low levels of virus, so although we know that HEV nucleic acid has been detected in food; it is not yet proven that this will be infectious [1,3].

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7. Appendix 1: Summary table of the food homogenates composition

Food Homogenates	Composition per	Energy	Fat	Saturates	Carbohydrates	Suga
Strawberry	100g	128kj/30kcal	0.1g	<0.1g	6.0g	6.0g
Raspberry	100g	133kj/32kcal	0.3g	0.1g	4.6g	4.6g
Lettuce	100g	48kj/11kcal	0.1g	<0.1g	1.4g	1.4g

Food Homogenates	Composition per	Energy	Fat	Saturates	Carbohydrates	Suga
Pork sausages (40% meat)	100g	460kj/240kcal	15g	5.5g	14g	1.2g
Pork sausages (75% meat)	100g	1182kj/288kcal	22g	7.6g	1.2g	0.1g
Pork chops	100g	1119kj/270kcal	21.7g	8.0g	0g	0g
Brussels Paté	100g	372kj/90kcal	7.1g	2.7g	0g	1.1g
Cooked Mussels	100g	401kj/95kcal	2.6g	0.5g	4.3g	0g
Raw Mussels	100g	86kcal	2.26g	0.4g	3.6g	0g
Raw oysters	100g	72kcal	1.5g	0g	3g	0g

Food Homogenates	Composition per	Calcium	Magnesium	Potassium	Sodium	Bicarbona
Bottled water	1 litre	27mg	10.5mg	3.5mg	35.3mg	211.8mg
Raw mussel	100g	26mg	-	320mg	286mg	-

Download the full data tables:

ODS
Gweld Appendix 1 Summary table of the food homogenates composition as ODS(Open in a new window) (3.56 KB)
ODS
Gweld Appendix 1 Summary table of the food homogenates composition (continued) as ODS(Open in a new window) (3.1 KB)

8. Appendix 2: Images depicting the viability of A549 (A), PLC (B) and HepG2 cells (C), after 72h following addition of

neat volume of various homogenates samples to the cells. Cells were seeded for 24h in 96 well plate at $1x10^4$ cells/well in DMEM:F12 supplemented with 10% FBS at 37°C in an atmosphere containing 5% CO_2 (x10).

A

B

C