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Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Infectious disease research doi: 10.1016/S2222-1808(16)61092-7

A novel porcine cell culture based protocol for the propagation of hepatitis E virus

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ARTICLE INFO

Article history:
Received 4 Jul 2016
Received in revised form 14 Jul, 2nd revised form 15 Jul 2016
Accepted 20 Jul 2016
Available online 26 Jul 2016

Keywords:
Porcine
Cell culture
Protocol
Propagation
Hepatitis E virus

ABSTRACT

Objective: To present a comprehensive protocol for the processing of hepatitis E virus (HEV) infected samples and propagation of the virus in primary cell cultures.
Methods: Hepatitis E was extracted from porcine liver and faecal samples following standard protocols. The virus was then allowed to attach in the presence of trypsin to primary cells that included porcine and bovine intestinal epithelial cells and macrophages over a period of up to 3 h. The virus was propagated by rotational passaging through the cell cultures. Propagation was confirmed by immunoblotting.
Results: We developed a comprehensive protocol to propagate HEV in porcine cell model that includes (i) rotational culturing of the virus between porcine cell types, (ii) pre-incubation of infected cells for 210 min, (iii) use of a semi-complete cell culture medium supplemented with trypsin (0.33 µg/mL) and (iv) the use of simple immunoblot technique to detect the amplified virus based on the open reading frame 2/3.
Conclusions: This protocol opens doors towards systematic analysis of the mechanisms that underlie the pathogenesis of HEV *in vitro*. Using our protocol, one can complete the propagation process within 6 to 9 d.

1. Introduction

Hepatitis E virus (HEV) is responsible for major outbreaks of acute hepatitis that have been recorded around the world, especially in developing countries including many parts of Africa and Asia[1]. Poor personal hygiene and water sanitation, together with tropical climates, have been blamed for the outbreaks of HEV that have occurred in many developing countries[2]. HEV has recently emerged as one of the major zoonotic and food-borne pathogens in developed countries, with sporadic cases having been associated with consumption of HEV contaminated pork liver sausages in France[3,4] or grilled/undercooked pig liver in Japan[5]. In Japan, 4 human cases of hepatitis E that occurred in 2003 were linked directly to consumption of raw deer meat[6]. Other cases of zoonotic transmission of HEV in Japan were linked to consumption of wild boar meat[7]. Several other cases of acute hepatitis E in humans have been epidemiologically linked to consumption of undercooked pork liver[3-5].

While mortality from HEV may be relatively low (approximately 1% in the general population), it is known to result in serious morbidity in children, young adults and pregnant women. Acute hepatitis E presents with clinical manifestations that are more indistinguishable than that of other acute viral hepatitis[1]. Hepatitis E disease is heralded by an abrupt onset of non-specific symptoms, followed by right upper quadrant pain, jaundice, anorexia, malaise, nausea and vomiting[1]. However, HEV infections are frequently asymptomatic, hence, it can go undetected especially in children[1].

Most HEV samples are collected from pigs exhibiting symptoms of the disease. Some surveys have demonstrated that prevalence of HEV in pigs may exceed 95%[8]. Replicative HEV has been isolated from small intest, lymph node, colon and liver samples of experimentally infected pigs[9]. Other animals such as the lesser bandicoot rat (*Bandicota bengalensis*), the Asian house shrew

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All experimental procedures involving animals were conducted in accordance to the Economic and Social Research Council (ESRC) Framework for Research Ethics (FRE) and approved by Framework Programme of the European Union (Contract number: Food-CT-2005-007081) as specified in the funding document.
Foundation Project: Surpported by European Union's Framework Programme Six (FP6), contract number: (Pathogen Combat, FP6-007081).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

(*Suncus murinus*), small Asian mongoose (*Herpestes javanicus*), common kestrel (*Falco tinnunculus*) and red-footed falcon (*Falco vespertinus*) in Central Europe, rats, bats, ferrets and rabbits can also act as reservoirs of HEV[10-16].

Laboratory animal care personnel, researchers, and support staff represent a new population at risk for HEV infection[16]. In general terms, laboratory-associated infections with HEV do not appear to be an important occupational risk among laboratory personnel[17]. The manipulation of HEV samples, faeces, blood, livers or other tissues from infected animals of HEV requires practices of biosafety level 2, containment equipment and facilities[17]. Despite all the evidence that HEV poses high risk to humans – in view of the possibility of zoonotic transmission and the potential of the virus to be used in bio-warfare, there is no single protocol to describe the successful propagation of HEV *in vitro* or *in vivo*[18]. A number of reports show that propagation of HEV is inefficient and limited[19]. Our attempts to understand the molecular mechanisms that underlie replication, pathogenesis and infection of HEV, frustrated by lack of a robust cell culture model for such studies[20]. As a result of these limitations, no vaccine or drug against HEV exists to date. The search of a cell culture model that is permissive for propagation of HEV has been a preoccupation of many scientists in recent years. The propagation of HEV in many cell culture models has largely been deemed grossly inefficient[21].

The swine HEV is a relatively new zoonotic agent which is closely related to the human HEV and is known to infect other non-human primates[22]. While pigs remain the primary zoonotic sources of HEV, human samples also may be collected and processed by research laboratories for propagation in cases of clinical trials, or in disease surveillance programs.

The genome of HEV is a polyadenylated, single-stranded, positive-sense RNA (approximately 7.2 kb), flanked with short non-coding regions at both the 5′ and the 3′ ends[23]. Further, the genome consists of three discontinuous and partially overlapping open reading frames (ORFs), namely, ORF1, 2 and 3-ORF 1, the largest of the three, encodes non-structural proteins including methyltransferase, protease, helicase, and RNA-dependent RNA polymerase[24]. The other two ORFs encode proteins of HEV, namely, pORF2 (a capsid protein) encoded by ORF2, and pORF3 (a phosphoprotein) encoded by ORF3 are used in various recombinant systems and they form the basis for diagnostic tests and vaccine studies[23]. The diagnosis of HEV in human and animal samples primarily relies on the immunodetection of pORF2 and pORF3, and immunoglobulins M (IgM) and immunoglobulins G (IgG)[25]. Techniques such as enzyme immunoassays, ELISA and Western blot assays are widely used to detect IgM and IgG anti-HEV antibodies in a variety of samples, while immunofluorescent antibody blocking assays are used to detect antibody to antigen of HEV in serum and liver[26]. IgM anti-HEV antibodies can be detected during the first few months after infection of HEV, whereas IgG anti-HEV antibodies represent either recent or remote exposure[26]. PCR is also widely used to detect HEV and RNA in serum and stool[27,28]. While RT-PCR can be used to detect HEV in biological samples. These assays are time consuming, inconvenient and cannot be used to quantify the virus[29]. Visualisation of the virus particles, particularly in faeces, is done through the use of immune electron microscopy[30,31].

To date, no single workflow incorporating with propagation of HEV has been described. However, a few reports demonstrating efficient propagation of the virus in cell culture and animal models are trickling in. Study of Jirintai *et al.* reported efficient propagation of rat HEV in PLC/PRF/5, HuH-7 and HepG2 cells, and its irrespective genetic group (G1-G3)[32]. Recently, study of van de Garde *et al.* described the successful propagation of HEV derived from infected individuals in a human-liver chimeric mouse model (uPA$^{+/+}$Nod-SCID-IL2R$\gamma^{-/-}$) next to a human pulmonary adenocarcinoma cell line (A549)[33]. Study of Shukla *et al.* showed that a virions of a quasispecies of a genotype 1 HEV (Sar55) and genotype 3 (Kernow) isolated from faeces were able to infect human HepG2/C3A hepatoma cells more efficiently than swine LLC-PK kidney cells[34].

Here, we present a comprehensive protocol for the processing of HEV infected samples and propagation of the virus in primary cell cultures derived from porcine small intestines (CLAB) and macrophages (POM-2) (Figure 1). Our protocol is comprised of a detailed description for preparation of reproducible sample which is extraction of HEV from pig faeces and livers, a multi-cell model for propagation of HEV, immunoassay (dot-blot) and RT-PCR based methods for qualitative and quantitative detection of the virus. The robustness of our protocol is demonstrated pictorially with pictures of a typical dot-blot and cytopathic effects in cell monolayers. This protocol can be adapted for different kinds of HEV bearing sample. This protocol has been validated internally since repeatable results were obtained. The proposed protocol remains to be validated by other laboratories.

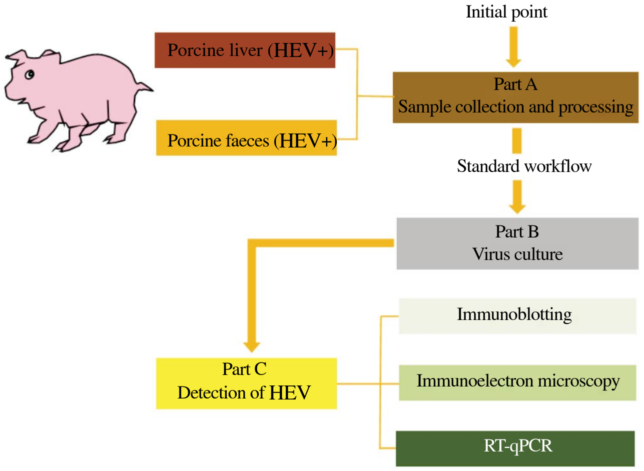


Figure 1. Protocol flowchart of isolation, culture and detection of HEV.

2. Materials and methods

Pig liver and faecal samples were obtained from the Swedish University of Agricultural Sciences. The presence of HEV in the pig samples was confirmed by PCR at Swedish University of Agricultural Sciences.

2.1. Extraction of HEV from liver tissue

The liver tissue was ground in a mortar in a small volume of phosphate buffered saline (PBS) (1 × PBS, 10% of volume/weight of tissue, 7.4 pH) supplemented with antibiotics, penicillin (100 IU/mL, Sigma-Aldrich) and streptomycin (1 mg/mL, Sigma-Aldrich). The supernatant of the liver suspension was transferred into a centrifuge tube and centrifuged at 3000 g under 4 °C for 30 min. Following the centrifugation, the supernatant was aliquoted into a number of eppendorf tubes and stored at –70 °C until the time for propagation or characterisation.

2.2. Extraction of HEV from pig faeces

A sample of the pig faeces was diluted in 1 × PBS solution (10% w/v) supplemented with antibiotics, penicillin (100 IU/mL, Sigma-Aldrich) and streptomycin (1 mg/mL, Sigma-Aldrich). The sample was homogenised, then centrifuged at 3000 g under 4 °C for 30 min. The supernatant was collected into a number of Eppendorf tubes and stored at –70 °C until the time for propagation or characterisation.

2.3. Maintenance of pig epithelial, macrophage and hepatic cells

The following mammalian cells were used in the propagation of HEV: (i) a primary pig small intestine epithelial cell culture (CLAB), which was isolated and maintained by University of Maribor, Slovenia, (ii) a primary pig macrophage cell culture (POM), which was isolated and maintained by University of Maribor, Slovenia, (iii) a primary bovine calf small intestine epithelial cell culture (CIEB) and (iv) a cell line derived from a human colonic adenocarcinoma, which differentiates into small intestinal-like cells after confluence (caco-2)[35]. The cells were grown in advanced Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich, Grand Island, USA), supplemented with 10% foetal calf serum (FCS) (Cambrex, Verviers, Belgium), l-glutamine (2 mmol/L, Sigma-Aldrich), penicillin (100 IU/mL, Sigma-Aldrich), and streptomycin (1 mg/mL, Fluka, Buchs, Switzerland) (complete cell culture medium) in 25 cm^2 culture flasks (Corning, New York, USA) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cell culture medium was changed after every 24–48 h. The cell culture medium was removed and the monolayer was washed with pre-warmed (room temperature) sterile 1 × PBS (7.2 pH). The cells were then harvested using a scrapper or trypsin.

2.4. Propagation of HEV porcine cells

The cells harvested from flasks, as described above, were re-suspended in complete cell culture medium. The cells were then counted using a haemocytometer under an inverted microscope. The cells were then seeded in 25 mL flasks in complete cell culture medium at a concentration of 6×10^6 cells/mL. The flasks were incubated at 37 °C in a humidified 5% CO_2 incubator until the monolayers were approximately 90% confluent (over a period of 24–48 h). The cell culture medium was removed and the monolayers were washed twice with 1 mL of pre-warmed 1 × PBS. About 100 µL of the virus suspension in DMEM supplemented with trypsin (0.33 µg/L), l-glutamine (2 mmol/L, Sigma-Aldrich, Grand Island, USA), penicillin (100 IU/mL, Sigma-Aldrich, Grand Island, USA) and streptomycin (1 mg/mL, Sigma-Aldrich, Grand Island, USA) was added to the cells, but without FCS was added to the cells. The flasks were incubated at 37 °C in a humidified 5% CO_2 incubator for 210 min. The cell culture medium was removed and the monolayers were washed twice with sterile 10% PBS. About 10 mL of fresh DMEM (Sigma-Aldrich, Grand Island, USA, Missouri, USA, or equivalent) supplemented with l-glutamine (2 mmol/L, Sigma-Aldrich, Grand Island, USA), penicillin (100 IU/mL, Sigma-Aldrich, Grand Island, USA) and streptomycin (1 mg/mL, Sigma-Aldrich, Grand Island, USA) was added to the wells. The flasks were incubated at 37 °C in a humidified 5% CO_2 incubator until cytopathic effects were evident/after a period of 24–48 h. The propagation of the virus was done using different cell combinations (*e.g.* propagation in CLAB cells followed by POM, and so on).

2.5. Determination of HEV titre

Determination of virus titre was done following the Reed and Muench protocol, which calculated 50% tissue culture infectious dose ($TCID_{50}$) of the virus. Briefly, the stock of virus suspension was diluted 10-fold to 10^{-2} in dilution buffer and stored as the working stock of the virus. The virus suspension was then diluted into complete cell culture medium on cell monolayers (in a 96 well plate) to 10^{-2}, $10^{-2.5}$, 10^{-3}… up to 10^{-7}. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator. After 24–48 h, the supernatants were removed from all wells. The cells were rinsed with pre-warmed 1 × PBS to remove cell debris. Upon washing, the plates were stained with 0.01% crystal violet for 5 min and then rinsed with water. The plates were then dried. The crystal violet incorporated in viable cells was re-suspended with 10% acetic acid (100 µL per well) and quantified with a microplate reader (Multiscan, Finland) at 595 nm. The $TCID_{50}$ was then calculated in accordance with the Reed and Muench protocol[36]:

$$TCID_{50} = \frac{\%\ \text{positive value above 50\%} - (50 \times 0.5)}{\%\ \text{positive value above 50\%} - \%\ \text{positive value below 50\%}}$$

2.6. Set-up of propagation experiment

The cell line including CLAB [primary cells from pig small intestine enterocytes (isolation protocol was available from Department of Biochemistry, Faculty of Medicine, University of Maribor, Maribor, Slovenia)]; PSI cl1 [primary cells from pig small intestine enterocytes, which were characteristically different from CLAB (isolation protocol was available from Department of Biochemistry, Faculty of Medicine, University of Maribor, Maribor, Slovenia)]; PSI cl3 [primary cells from pig small intestine enterocytes, which were characteristically different from CLAB (isolation protocol was available from Department of Biochemistry, Faculty of Medicine, University of Maribor, Maribor, Slovenia)]; CIEB [primary cells of human macrophage origin (isolation protocol was available from Department of Biochemistry, Faculty of Medicine, University of Maribor, Maribor, Slovenia)] and Caco-2 (a cell line derived from a human colonic adenocarcinoma, but it differentiates into small intestinal-like cells after confluence) (Table 1)[35].

Table 1
Time for HEV to cause cytopathic effect (CPE) on different cell lines (first exposure to HEV).

Cell line	CPE (hours of post infection)	
	Liver tissue sample	Faecal sample
CLAB	24	48
PSI cl1	48	48
PSI cl3	24	24
CIEB	24	24
Caco-2	No CPE	No CPE

2.7. Immunodetection of HEV

Porcine cells previously grown to confluency in T25 flasks were seeded in 24 well plates at a concentration of 6 × 10^6 cells/mL in complete cell culture medium. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator until the monolayers were approximately 90% confluent (over a period of 24–48 h). The growth medium was removed from the flasks and the cells were washed twice with pre-warmed (20–25 °C) sterile 1 × PBS (7.2 pH). Aliquots of the virus suspension (1 000 μL) (Table 2) in DMEM supplemented with trypsin (0.33 μg/L), l-glutamine (2 mmol/L, Sigma-Aldrich, Grand Island, USA), penicillin (100 IU/mL, Sigma-Aldrich, Grand Island, USA) and streptomycin (1 mg/mL, Sigma-Aldrich, Grand Island, USA), but without FCS were added to the cells. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator for 210 min. The cell culture medium was removed, then the monolayers were washed twice with sterile 10% PBS. About 1 000 μL of fresh DMEM (Sigma-Aldrich, Missouri, USA) supplemented with l-glutamine (2 mmol/L, Sigma-Aldrich, Grand Island, USA), penicillin (100 IU/mL, Sigma-Aldrich, Grand Island, USA) and streptomycin (1 mg/mL, Sigma-Aldrich, Grand Island, USA) was added to each well. The plates were incubated at 37 °C in a humidified 5% CO_2 incubator until cytopathic effects of the virus were observed (over a period of 24–48 h). When CPE was observed, the cell monolayers were washed twice with DMEM without phenol red and supplements. Immunoblotting for the presence of HEV was conducted using Anti-HEV ORF2.1 Antibody, clone 4B2 (Sigma-Aldrich, Grand Island, USA) against Anti-Swine IgG (H+L)-Peroxidase antibody produced in goat (Sigma-Aldrich, Grand Island, USA) (1:5000 dilution), following the protocol described by Bio-Rad Laboratories (Marnes-la-Coquette, France). In this method, the intensity of the dots can be used to qualitatively assess the extent of propagation of HEV.

Table 2
Propagation setup in P96 well plates.

Sequence	Samples								Controls without first antibody			
	1	2	3	4	5	6	7	8	9	10	11	12
A	Control[a]	Control[a]	HEV + faecal material[a]	HEV + faecal material[a]	HEV + LIV G.CNT[a]	HEV + LIV G.CNT[a]	HEV + LIV-TRYP[a]	HEV + LIV-TRYP[a]	Control[a]	HEV + faecal material[a]	HEV + LIV[a]	HEV + LIV[a]
B	HEV + KIDN[a]	HEV + KIDN[a]	TRYP CLAB 37 °C[a]	TRYP CLAB 37 °C[a]	TRYP CLAB 37 °C[a]	TRYP CLAB 37 °C[a]	HEV + faecal material[a]	HEV + faecal material[a]	HEV + KIDN[a]	TRYP CLAB 37 °C[a]	TRYP CLAB 37 °C[a]	HEV + faecal material[a]
C	TRYP CLAB 40 °C[a]	TRYP CLAB 40 °C[a]	TRYP CLAB 37 °C[b]	TRYP CLAB 37 °C[b]	TRYP CLAB 40 °C[b]	TRYP CLAB 40 °C[b]	Control[c]	Control[c]	TRYP CLAB 40 °C[a]	TRYP CLAB 37 °C[b]	TRYP CLAB 40 °C[b]	Control[c]
D	HEV + LIV[c]	HEV + LIV[c]	HEV + LIV[c]	HEV + LIV[c]	HEV + KIDN[c]	HEV + KIDN[c]	TRYP CLAB 37 °C[c]	TRYP CLAB 37 °C[c]	HEV + LIV[c]	HEV + LIV[c]	HEV + KIDN[c]	TRYP CLAB 37 °C[c]
E	TRYP CLAB 37 °C[c]	TRYP CLAB 37 °C[c]	HEV + faecal material[c]	HEV + faecal material[c]	TRYP CLAB 40 °C[c]	TRYP CLAB 40 °C[c]	Control[b]	Control[b]	TRYP CLAB 37 °C[c]	HEV + faecal material[c]	TRYP CLAB 40 °C[c]	Control[b]
F	HEV + LIV[b]	HEV + LIV[b]	HEV + LIV[b]	HEV + LIV[b]	HEV + KIDN[b]	HEV + KIDN[b]	TRYP CLAB 37 °C[b]	TRYP CLAB 37 °C[b]	HEV + LIV[b]	HEV + LIV[b]	HEV + KIDN[b]	TRYP CLAB 37 °C[b]
G	TRYP CLAB 37 °C[b]	TRYP CLAB 37 °C[b]	HEV + faecal material[b]	HEV + faecal material[b]	TRYP CLAB 40 °C[b]	TRYP CLAB 40 °C[b]	Control[d]	Control[d]	TRYP CLAB 37 °C[b]	HEV + faecal material[b]	TRYP CLAB 40 °C[b]	Control[d]
H	HEV + LIV[d]	HEV + LIV[d]	HEV + LIV[d]	HEV + LIV[d]	HEV + KIDN[d]	HEV + KIDN[d]	HEV + faecal material[d]	HEV + faecal material[d]	HEV + LIV[d]	HEV + LIV[d]	HEV + KIDN[d]	HEV + faecal material[d]

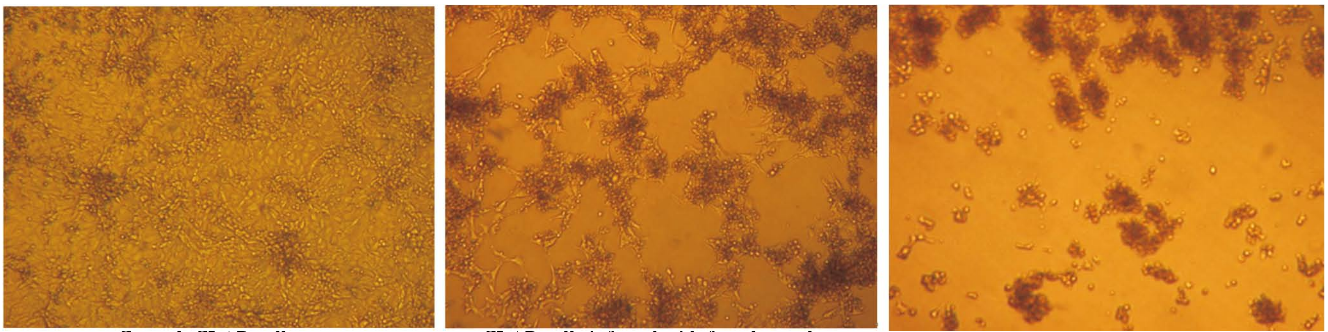
Control: Cell culture only; HEV + faecal material: HEV suspension from pig faecal material; HEV + LIV-TRYP: HEV suspension from liver tissue in PBS 1 × (50 μL) + DMEM medium with trypsin (50 μL); HEV + LIV: HEV suspension from liver tissue in DMEM medium with trypsin (50 μL); G.CNT: HEV suspension from liver tissue in PBS 1 × (50 μL) + gut content (50 μL); HEV + KIDN: HEV suspension from kidney in PBS 1 × (50 μL); TRYP CLAB 37 °C: HEV suspension from liver tissue in PBS 1 × (50 μL) + DMEM medium with trypsin (50 μL) incubated at 37 °C; TRYP CLAB 40°C: HEV suspension from liver tissue in PBS 1 × (50 μL) + DMEM medium with trypsin (50 μL) incubated at 40 °C; F12: Faecal in PBS 1 × with ATB (50 μL) + gut content (50 μL); [a]: CLAB with virus for 3h and 30 min; [b]: POM with virus for 3h and 30 min; [c]: POM with virus for 1h and 30 min; [d]: CLAB with virus for 1h and 30 min.

In addition, for the purposes of comparison, detection of anti-HEV IgG antibodies in the cell supernatants against Anti-Swine IgG (H+L)-Peroxidase antibody (Sigma-Aldrich, Grand Island, USA) produced in goat (1:5000 dilution) as a secondary antibody was also done in accordance with the protocol described by Bio-Rad Laboratories. A conjugated anti-swine IgG was used as a secondary antibody.

3. Results

3.1. CPE of HEV in a primary mammalian cell culture model

CPE of HEV extracted from faecal/liver samples was shown on CLAB and POM-2 cells (Figure 2).



Control: CLAB cells
CLAB cells infected with faecal sample
CLAB infected with cell supernatant from caco-2 cells (2nd passage of the virus)

Figure 2. CPE on CLAB and caco-2 cell lines after infection with samples containing HEV.

3.2. Immunoblotting for HEV following a propagation protocol

CLAB and POM-2 were permissive for propagation of HEV, but only when the cells were infected with a sample of HEV from the liver of the infected pig. The qualitative assessment of the depth of the dots showed that the addition of trypsin may have increased the titre of the virus in both CLAB and POM-2. However, caco-2 cells were not permissive to propagation of HEV (Figure 3).

This result illustrated the need for a pre-incubation time of 210 min to allow the virus to attach. Supernatants of HEV derived from pig liver that were propagated in porcine enterocytes (CLAB) or porcine macrophages (POM) cells for 210 min, but not for 1 h, showed the evidence of efficient propagation as shown by dots in box B following immunoblotting (Figure 4).

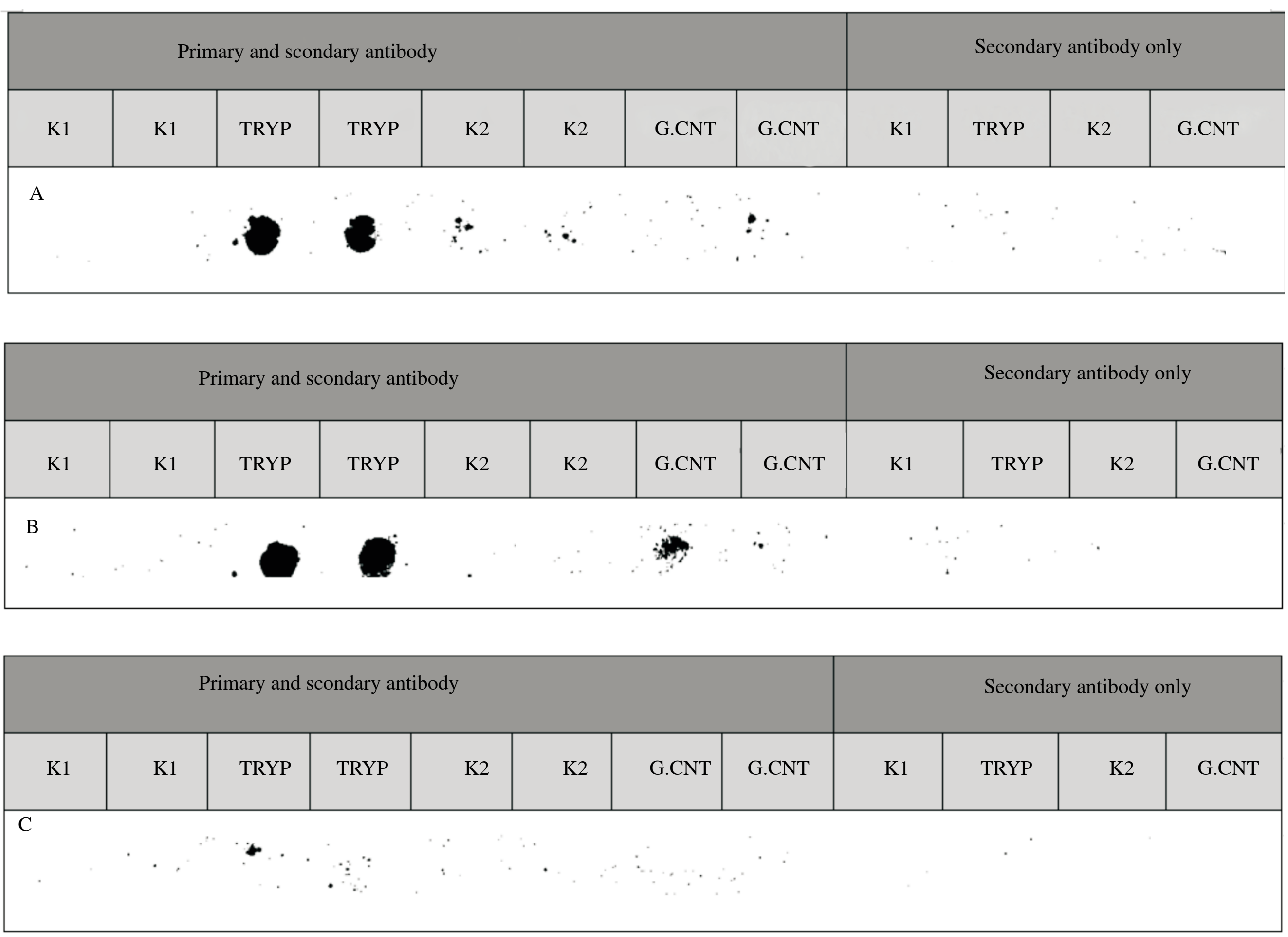


Figure 3. Immunoblot of HEV in cell supernatants of porcine cell culture exposed to different culture conditions.
A: CLAB cells infected with HEV virus from an infected pig; B: POM-2 cells infected with HEV virus from an infected pig; C: Caco-2 cells infected with HEV virus from an infected pig. K1: Control 1 (CLAB cells not infected with HEV); K2: Control 2 (cell culture medium not infected with HEV); TRYP: CLAB cells infected with HEV from liver samples, with trypsin added; G.CNT: CLAB cells infected with HEV from liver tissue in PBS 1 × (50 µL) + gut content (50 µL).

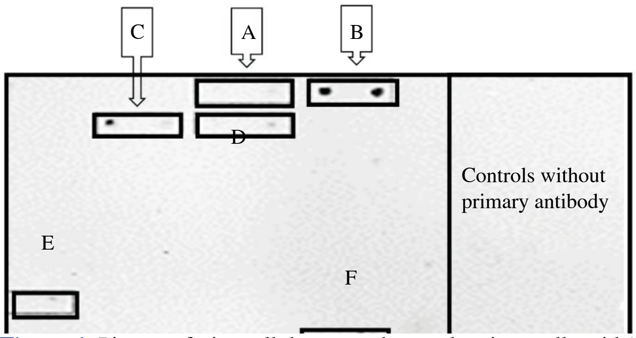


Figure 4. Picture of nitrocellulose membrane showing wells with/without detectable HEV using dot-blot technique

A: HEV originating from pig liver, propagated in CLAB cells, following a pre-incubation period of 210 min in complete cell culture medium (without FCS) supplemented with gut content; B: HEV originating from pig liver, propagated in CLAB cells, following a pre-incubation period of 210 min in complete cell culture medium (without FCS) supplemented with 0.33 µg/L of trypsin; C: HEV originating from pig liver, propagated in CLAB cells (twice), following a pre-incubation period of 210 min in complete cell culture medium (without FCS) supplemented with 0.33 µg/L of trypsin; D: HEV originating from pig liver, propagated in CLAB cells (twice), following a pre-incubation period of 210 min in complete cell culture medium (without FCS) supplemented with 0.33 µg/L of trypsin; E: HEV originating from pig liver, propagated in POM cells (twice), following a pre-incubation period of 210 min in complete cell culture medium (without FCS) supplemented with 0.33 µg/L of trypsin; F: HEV originating from pig liver, propagated in CLAB cells (twice), following a pre-incubation period of 210 min in complete cell culture medium (without FCS) supplemented with 0.33 µg/L of trypsin.

4. Discussion

We have described the first protocol for efficient propagation of HEV in a porcine cell culture model, albeit without validation. Many protocols specify the need for a pre-incubation of 1 h to allow attachment and entry of viruses other than HEV into cells. Examples of such viruses requiring a pre-incubation step lasting 1 h include herpes simplex virus, rotavirus and so forth[37,38]. Here we demonstrate that, for HEV to propagate in porcine cells (CLAB/POM), there is need for pre-incubation of the cell-virus culture for 210 min, and supplementation of the cell culture medium (DMEM) with trypsin (0.33 µg/L), L-glutamine (2 mmol/L, Sigma-Aldrich, Grand Island, USA), penicillin (100 units/mL, Sigma-Aldrich, Grand Island, USA) and streptomycin (1 mg/mL, Sigma-Aldrich, Grand Island, USA) (but without FCS). The ability of HEV to propagate in the porcine cells was demonstrated through at least three trials, where the virus was detectable using dot-blot after incubation for 210 min, but not for 60 min.

The use of trypsin to enhance attachment/entry of HEV has not been described prior to our research. Based on our findings, the mechanisms by which trypsin enhances the propagation of HEV remain unknown. Trypsin has been shown to enhance postattachment entry of rotavirus, and its growth in a monkey kidney cell line (MA104)[39,40]. The activation of rotavirus entry into MA104 cells was shown to be associated with cleavage of the viral haemagglutinin (viral protein 3) into two fragments (60 and 28 kilodaltons)[39].

In summary, we have established the first ever propagation protocol for HEV in a porcine cell culture model. Subsequent inter-laboratory validation of the protocol is necessary. Rotational incubation of HEV in different porcine cell cultures, together with a pre-incubation of the virus on porcine cells for 210 min, in complete cell culture medium supplemented with 0.33 µg/L of trypsin, were shown to be effective to propagate HEV as detected by the dot-blot technique. Trypsin increases the attachment/entry of the virus.

The HEV propagation workflows derive from lack of a quantitative immunological assay method. Reliance on dot-blot has several limitations. Firstly, internal validation of the method and quality control are necessary in every laboratory. The novelty of immunoassays is limited by the high chances of contamination, which is characteristic of the technique. Other challenges concern the choice of reagents/materials for use in the tests. Reagents from different manufacturers may have varying levels of purity, hence the selection of the right materials is essential. Attempts were made to specify the materials that were used during the establishment of the protocol below.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

This research was conducted under Pathogen Combat (FP6-007081), a consortium that was funded under a Framework 6th Programme of the European Union. Pig liver and faecal samples were provided by Swedish University of Agricultural Sciences (SLU). A great thank you goes to colleagues at University of Maribor who contributed indirectly to this work.

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