ONLINE SUPPLEMENT

Porcine-Derived Pancreatic Enzyme Replacement Therapy May Be Linked to Chronic Hepatitis E Virus Infection in Cystic Fibrosis Lung Transplant Recipients

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SUPPLEMENTARY METHODS:

Hepatitis E Serological Testing of Cohorts

Patient serum was collected in 5 ml vacutainers and processed using standard protocols by Alberta Precision Laboratories clinical staff.

HEV Serological Testing - ELISA Details

HEV IgG and IgM were assessed from CF transplant recipients at the Canadian National Microbiology Laboratory using an ELISA assay from Wantai Biopharm (Beijing, China) as part of their clinical care. From the non-transplant CF prospectively enrolled research cohort, all samples were screened first with the Abbexa Ca. ABX364866 ELISA assay, following manufacturer's instructions. Samples that were positive were confirmed with the Elabscience Biotechnology Ca. E-HD-E055 ELISA assay, following manufacturer's instructions to ensure seropositivity rates were not overestimated.

Extraction of RNA from PERT

In order to mitigate the interference of the enteric coating present in most formulations of PERT, capsules were dissolved in 1 to 3 ml of 2% sodium bicarbonate at room temperature for up to 1 hour [1] supplemented with 2 µl RNaseOut (Life Technologies Ca 10777019) and a spiked exogenous positive control [Calf Guard dissolved in 1mL PBS; 5ul Bovine Coronavirus (BCoV)]. Once dissolved, PERT samples were aliquoted into 0.5 ml aliquots. TRIzol™ was used to better purify RNA from the exceedingly protein rich, enzymatically active PERT matrix, and to mitigate the effects of enteric coating. One milliliter of TRIzol™ was mixed with dissolved aliquots as per the TRIzol™ reagent instruction for RNA purification by vortexing for

15 seconds followed by the addition of 200 µl Chloroform and vortexed for 15 seconds. Samples were then centrifuged for 10 min at 14000 rpm. The aqueous phase was carefully pipetted into another 200 µl chloroform, mixed well and centrifuged for 10 min at 14000 rpm. The aqueous phase was extracted and added to 500 µl of 100% EtOH followed by incubation at room temperature for 5 minutes. Each of the aliquots derived from the same PERT capsule were pooled and then processed using QIAamp MinElute Virus spin (Qiagen Ca. 57705) columns with 1 column for each 1 ml of dissolved sample. Columns were rinsed with AW1 buffer and 80 µl of Turbo DNase (Life Technologies Ca. AM2238) (10 µl enzyme 2U/ µl, in 70 µl buffer) was added to the column and incubated at room temperature for 15 minutes. Columns were washed again with 500 µl AW1 buffer followed by 700 µl AW2 and 700 µl of 100% EtOH, then dried and eluted in 50 µl Ultra-Pure water (Life Technologies). All samples were run alongside negative controls of 2% sodium bicarbonate buffer, 2 µl RNaseOut, and 5 µl Calf Guard with reagents only.

HEV Quantification by RTqPCR and RTdPCR

One step RTqPCR was performed in duplicate using 5 µl of extracted samples with Taqman Fast virus 1-step master mix (Life Technologies Ca. 4444432), 250 nM primers and 200 nM probe as described previously [2] in a final volume of 20 µl to amplify the *orf3* target sequence. Serial dilutions from $5x10^6$ to 0.5 GC of a gBlock (IDT) modified from Salvio *et al* (2) were used for the standard curve. Each run included standard negative controls and extraction negative controls. RTqPCR thermal cycle conditions for the master mix were as follows and preformed on the QuantStudio5 (Applied Biosystems): Reverse transcription 50°C for 5 minutes, then 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds and 60°C for 30 seconds. Data was

analysed on the QuantStudio Design & Analysis Software version 1.5.2. Samples within a threshold of ≤42 cycling times were considered positive. Owing to the very high rates of PCR inhibitors in porcine-derived, highly proteinaceous PERT, each sample was assessed a second time in duplicate using a 1/10 dilution of the original extracted sample. Samples were deemed positive if either the primary or 1/10 dilution had HEV RNA detected with a RT-qPCR quantification cycle (Cq) value of ≤42. All molecular primers used in the study are available in ***Supplemental Table 1.*** Both buffer extraction controls and RTqPCR negative control (reagents and water only) were run for each extraction batch and RTqPCR plate, respectively.

One step RT-digital PCR was done using the Absolute Q 1-step RT-dPCR Master Mix (Life Technologies Ca. A55146) according to manufactures instructions on the QuantStudio™ Absolute Q™ Digital PCR System (Applied Biosystems). In brief, 2.5 µl 4x master mix was mixed with 250 nM each primer and 400 nM probe used for RTqPCR [2] and added to a single tube where 1 µl of extracted sample was added to a final volume of 10 µl . Thereafter, 9 µl of sample with master mix was added to one of 16 wells of the QuantStudio™ Absolute Q™ MAP16 plate, followed by 15 µl of Absolute Q isolation buffer (A52730). Cycle conditions were as follows: reverse transcription 55°C for 10 minutes, 96°C for 10 minutes, 45 cycles of 96°C for 5 seconds and 60°C for 10 seconds. A negative template control (reagents and water only) and positive template control consisting of a 100 GC gBlock were also used for RTqPCR and ran on every plate. Samples negative on RTdPCR but positive on RTqPCR were repeated. Baseline threshold was set with negative control for each run. RTdPCR data was analysed on QuantStudio Absolute Q Digital PCR software version 6.

Use of an exogenous Positive Control - BCoV

Bovine coronavirus (BCoV) was used as an exogenous positive control. One step RTqPCR was performed to validate the BCoV spike (Calf Guard) using 5 µl of extracted samples with Taqman Fast virus 1-step master mix (Life Technologies Ca. 4444432), 200 nM primers and 125 nM probe as described previously [3] in a final volume of 20 µl. Tenfold dilutions starting at $5x10^8$ to 50 GC of target gene in a plasmid were used for creating the standard curve. For each PERT extraction, a buffer blank with BCoV spike was assessed in parallel. Owing to the very high rates of PCR inhibitors in porcine derived PERT, each sample was assessed using both the original 5 µl of extracted sample, and a second 1/10 dilution performed in duplicate. The Cq of BCoV spiked buffer blank and BCoV spiked samples were compared for inhibitors. If the difference in Cq between spiked samples and buffer was >2 Cq, samples were considered to contain inhibitors as described previously.[4]

Nested PCR and Sanger Sequencing of HEV RNA

Nested PCR-1:

HEV *orf1* targeting the regon **4228-4565** (**Figure 2**) [5] is perfomed by the public health agency on all swine and human HEV samples from across Canada. In brief, extracted RNA was eluted into 50 µL and amplified using hemi-nested, broadly reactive primers. The final 337 bp amplicon product was purified and cycle sequenced using an Applied Biosystems 3730 XL DNA Analyzer (ThermoFisher Scientific, Mississauga, ON) with nested primers. The research lab employed the same nested PCR-1 as above to compare with public health sequence data. Reverse transcription was performed using Superscript IV (Life Technologies Ca.1809005) with 0.5 µl, 1 µl and 5 µl of extracted PERT capsule samples, using Random Hexamer primers (Life technology Ca.

N8080127) and RNaseOut in a 20 µl final volume. Nested PCR was performed on 2 µl cDNA using Platinum Taq polymerase (ThermoFisher Scientific Ca 15966025) as in Drexler, *et al* [5] in a 25 µl final volume on all sequenced samples for PCR-1 *orf1*. Nested PCR second reaction used the same conditions with 1 µl from the first PCR and primers for nested PCR second reaction. Samples were run on a 1% agarose gel and appropriate size bands were cut out and purified using the QIAquick gel purification kit (Qiagen Ca 28706). 60 ng gel purified sample and 5 pmol primer were used for Sanger sequencing.

Nested PCR-2 and 3:

Two different, additional targets were amplified using nested PCR for further verification. Nested PCR-2 (*orf2* [6] gene target 5622-5911) and PCR-3 (*orf1* [7] gene target 22-561) (**Figure 2**) on several samples were also PCR amplified with Phusion polymerase (Life Tech Ca. F630S) as per the manufacturer's instructions with 500 nM primers with cycles as described previously.[5-7]

HEV RNA Genotyping

Sequencing file traces were assessed and trimmed using Benchling (https://www.benchling.com/) and uploaded to the Hepatitis E Genotyping Tool (https://www.rivm.nl/mpf/typingtool/hev/how-to-use) as described previously.[8] All PERT HEV genotyping was performed under sterile conditions in the university research laboratory whereas all patient samples were sequenced in the in the National Microbiology Laboratory (with the exception of Case 2 which was sequenced in both). Phylogenetic analysis of output and

reference sequences was performed by maximum likelihood inference of a 315 bp trimmed *orf1* alignment using DIVEIN web tools [9] by the TN93+γ+I model.[10]

HEV Whole Genome Sequencing from PERT

Long Range PCR

PERT was dissolved and RNA extracted as described in the main text. Concentrated samples were made pooling RNA extractions for 3 enzymes with 1 column each, final elution volume of 150 µL. A control using RNA from Patient 2 plasma was extracted using 400 µL plasma and 800 µL s for buffers with carrier RNA for the QIAamp MinElute Virus spin kit (Ca. 57705). RNA (0.5 to 5 µL) was used to make cDNA with 50 µM Oligo dT (Theremofisher Ca SO131) or 50 ng random hexamer primers (Thermofisher Ca.SO142) using Superscript IV (ThermoFisher Ca. 18090050) as directed by the manufacture's instruction with RNAase out (ThermoFisher Ca 10777019). Time and temperature for reverse transcription was modified to 60°C for 20 min as described previously.[11] Each round of Long Range PCR used nested PCR primers obtained from Papp *et al* [11] was carried out using Platinum™ SuperFi™ (ThermoFisher Ca.12351010) lrPCRF. Master mix was prepared using 5 µL Superfi Buffer, 0.5 µL 10mM dNTP's, 1 µL 10 µM of each primer, 1 to 2 µL template, 5 µL 5x GC enhancer and 0.25 µL SuperFi™ polymerases in a final volume of 25 µL.[12] Cycle conditions were as follows: 95°C for 3min, 10 cycles of 98°C for 10 seconds, 72°C decreasing by 1 degree per cycle for 10 seconds and 72°C for 4 minutes, 35 cycles of 98°C for 10 seconds, 68°C for 10 seconds and 72°C followed by 72°C for 8 minutes. PCR products were visualized on a 1% agarose gel stained with GelRed (Biotium, Fremont, CA. Ca. 41003) Bio-Rad ChemiDoc Touch. Primers used for sequencing are listed in ***Supplemental Table 2.***

Overlapping Primers

RNA extractions and cDNA were made as above with all cDNA and RNA samples positive for Nested PCR tested. Platinum™ SuperFi™ (ThermoFisher Ca.12351010) polymerase was used with primers as described previously.[13] Master mix was prepared as described earlier. Cycle conditions were as follows: 95°C for 3 minutes with 10 cycles of 98°C for 15 seconds, 68°C and decreasing by one degree per cycle, 72°C for 1:30 minutes, followed by 35 cycles of 98°C for 15 sec, 61°C for 10 sec, 72°C for 1:30 minutes with a final 72°C extension for 5 minutes. Samples were visualized on a 1% agarose gel stained with Gel Red on Bio-Rad ChemiDoc Touch. Primers used for sequencing are listed in ***Supplemental Table 2***.

Direct cDNA Sequencing using a Nanopore Long-Read Approach

PERT was dissolved and RNA extracted as described above. cDNA was synthesised as in the Oxford Nanopore protocol for ligation sequencing V14 – Direct cDNA sequencing (SDK-LSK114) after double stranded cDNA was prepared using switch strand primers, the Ligation sequencing amplicon – Native Barcoding Kit 24 V14 (SQK-NBD114.24) was used for barcoding and library preparation. The library was sequenced on the MinION flow cell FLO-MIN114 R10 for a total of 43 hours. Primers used for sequencing are listed in ***Supplemental Table 2***.

Mitigation of PERT Matrix Interference

The PERT matrix contains exceptionally high concentrations of enzymatically active proteins in each capsule with a range of active units including: protease, lipase, amylase, pH sensitive enteric coating, other pancreatic enzymes (i.e., RNase)[14] and other proprietary pharmaceutical

substances). To dissolve enterically coated capsules, several buffers were tested including 2% and 8% sodium bicarbonate, PBS (Phosphate buffered saline), 1M sodium acetate pH 7, TE (10mM Tris-HCl pH7.5, 1mM EDTA) and 0.1 M sodium phosphate (pH 8). Ultimately, 2% sodium bicarbonate and 0.1 M sodium phosphate (pH 8) were selected based on efficacy. We tested pH effects using 0.1M sodium phosphate buffer pH 6.8 and pH 8.0 on samples from two of the enterically coated PERT formulations. For RNA extractions, Trizol™ was used to circumvent enzymatically active protein rich substrate. Concentration of samples was done by pooling Trizol™ extractions into one Qiagen viral spin column. Attempts were made to concentrate and purify whole virions from PERT matrix using 100kDa ultrafiltration.[15] Ten PERT capsules from the same lot and manufacture were dissolved in 20 ml 2% sodium bicarbonate for one hour at room temperature followed by centrifugation at 4000 rpm for 30 minutes. The supernatant was then transferred to an ultracentrifuge tube and centrifuged at 20,000 rpm for 60 minutes. Following this, the supernatant was added to an Amicon or Centricon 100kDa concentrator (UFC710008) and centrifuged at 3000 rpm for up to 3 hours. Enterically coated samples were noted to clog filters with all applications with filtrate and concentrate volumes recorded. To attenuate concentrators from clogging, we attempted removal of enteric coating by precipitation at lower pH values. Enteric and non-enteric coated samples were dissolved in 2% sodium bicarbonate, spun down at 4000 rpm for 30 minutes followed by 20,000 rpm for 60 minutes with small aliquots of 1N HCl added. The pH was tested and any precipitate formation was recorded. Samples were then spun down again and filtered through 0.45 *u*M filter and concentrated in 100kDa concentrators.

HEV Protein Assessment from PERT

Samples were prepared as above to concentrated 10 PERT capsules. The concentrate was mixed 1:1 with 50 µL2x Laemmli sample buffer +/- 200mM dithiothreitol (DTT), then heated at 95°C for 2 min. If samples were not heated the protein ladder degraded from active proteases in the PERT extracts. For SDS page, 5 to 15 µL of sample was ran on 1mm thick 10-12% acrylamide gel. To prepare samples for mass spectrometry, 1 mm thick 10% SDS page gel was run for 30 minutes at 150V with the section cut out between the top of ladder and ~60kDa (5mm by 5mm). The portion was then rinsed three times in ultra-pure water and stored at 4°C. Western blots were transfer from SDS-page method above, 5ul-15ul concentrate and filtrate of PERT concentrate samples were assessed. Multiple positive controls were attempted (each using plasma from Case 2); PERT spiked with HEV plasma concentrate, buffer spiked with HEV plasma concentrate, confirmed HEV positive tissue culture supernatant. Negative controls utilized plasma from HEV seronegative individuals, 100mg/400ul PBS homogenized pork pancreas and negative tissue culture supernatant. Proteins on SDS page were transferred using Bio-Rad dry blotter 8min 2.5V in 1x Bio-Rad Trans-BlotTurbo Transfer buffer (Ca.10026938) to 0.45uM nitrocellulose. Blots were blocked in TBST (0.2M Tris pH 7.4, 1.5M NaCl2, 0.1% tween 20), 5% skim milk, overnight 4°C with rocking. Primary HEV Capsid ORF2 mouse antibody from Abcam (Ca. AB167453) and Sigma (Ca. MAB8002) were used at 1:1000 dilution in TBST, 5% skim milk, rotating for 1 hour at room temperature. This was followed by 3x washes in TBST for 10 minutes each. Secondary anti mouse IgG HRP antibody (Abcam Ca. AB6789) was used at 1:10000 dilution in TBST 5% milk and incubated for 1 hour at room temperature. The Blot was washed 3x in TBST for 10 min each. One mL of ECL reagent was mixed 1:1 (Sigma Ca. WBKLS0500) applied to blot and image on Chemi-Doc (Bio-Rad), as previously described.[16]

HEV Cultivation from PERT by Cell Culture

Cell culture methods were adapted from Schemmerer *et al* [17] using the cell lines A549, HepG2 and HuH-7. Cell lines were cultured in BMEM (Eagle minimum essential medium [MEM]) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, 1% non-essential amino acids (NEAA), 100 U/mL penicillin G and 100 µg/mL streptomycin and MEMM (BMEM additionally supplemented with 2.5 µg/mL amphotericin B and 30 mM $MgCl_2$). Cell cultures were seeded at concentrations of $10^5/cm^2$ viable cells in T25 flasks or 6 well plates in BMEM. Cell lines were then switched to MMEM and grown for 14 days prior to inoculation and cultured at 37°C with 5% CO_2. HepG2 cells were inoculated with dissolved PERT from each manufacturer, positive plasma control and negative PBS buffer control. Samples were dissolved in 1 ml 2% sodium bicarbonate, vortexed and stirred with a sterile loop. PBS 0.2% BSA (Filter sterilized) was added and vortexed. Half of each sample was heated to 50°C for 5min and then vortexed followed by centrifugation at 8000 x g for 30 min. The supernatant was then filtered through a 0.45 to 0.2um PES filter with media removed and 250 µL of inoculum including buffer control and positive plasma control added (filtered 0.2uM filter). These were then incubated at room temperature for 75 min. Thereafter, 2.5 ml of media was added and incubated at 34°C with 5% CO2. Media was completely refreshed at 24 hours and every 3-4 days thereafter. Supernatant was collected and RTqPCR was preformed to check for positive tissue cultures. Cells were split on day 15 to check by immunofluorescence. A549, HepG2 and HuH-7 cell lines were inoculated with Dynabead Intact Virus enrichment from PERT and HEV positive plasma. Two PERT enzyme capsules from each manufacture were dissolved in 1 ml 2% sodium bicarb and then incubated for up to one hour followed by vortexing and once dissolved, addition of PBS (3 mL) was completed. A negative buffer control and a positive control containing 500

240 µL positive patient plasma was added to the same buffers and extracted. Samples were spun down in 2 ml microfuge tubes at 8000xg for 30 min. Dynabead Intact Virus Enrichment (positively charged magnetic beads ThermoFisher Ca 10700D) manufactures protocol was followed and 80 µL was added to the 4 ml PERT solution. Samples were incubated on a rotator for 10 minutes at room temperature then applied to a magnetic stand for 1 minute with 245 supernatant removed. Dynabeads were rinsed with 1 ml PBS and mixed thoroughly, applied to a magnet for 1 minute with supernatant removed for up to two times or until beads were clear. To elute, 500 µL release buffer (50mM Citric Acid, 50mM sodium phosphate) was added and incubated rotating for 10 min at room temperature. Samples were applied to a magnet for 1 minute and the supernatant transferred to a new tube. The buffer was exchanged to PBS 0.2% 250 BSA using a 10kDa concentrator to a final volume of 1500 µL for infecting the 3 different cells lines. The media in the 6-well tissue culture plates was removed and 250 µL extract per well was added for 75 minutes at room temperature. Media (2.5 mL) was added to wells and incubated at 34.0°C with 5% CO_2. The media was completely refreshed at 24 hours and every 3-4 days thereafter. Supernatant was collected and RTqPCR was preformed to check for positive tissue 255 culture. Cells were split on day 15 to check by immunofluorescence.

SUPPLEMENTARY RESULTS

HEV Serological Testing - ELISA Details

260 Twenty-three percent of samples (n=19/83) were positive via Abexxa, and 84% of samples (n=16/19) of these were confirmed positive with the Elab assay. Subjects with discordant HEV ELISA results were classified as seronegative.

Assessment of PERT for Inhibition

We compared quantified BCoV between each spiked blank and the corresponding PERT and observed large discrepancies in cycles quantified, confirming high rates of PCR inhibitors in extracted PERT samples (***Supplemental Table 3***). As described by Ahmed and colleagues,[4] a delta Cq > 2 between BCoV spiked sample and BCoV spiked buffer indicates presence of inhibitors. Indeed in our study, 71% of samples had a delta Cq > 2 and Cq improved for most samples with a 1:10 dilution. Forty three percent (46/107; 43%) of samples Cq for BCoV improved with a 1 in 10 dilution. Twenty five percent (n=27/107; 25%) of samples had a Cq difference between the sample and the buffer spiked control of >10, suggesting high levels of PCR inhibition that may prevent HEV detection.[4]

Comparing HEV Quantification by RTqPCR and RT-dPCR

PERT samples assessed by RTqPCR worked best when diluted 1:10 as opposed to undiluted. These findings, along with the BCoV spike experiment, suggested the presence of inhibitors. Forty seven of 107 (47%) RTqPCR samples positive for HEV yielded a median 50 copies (cp)/capsule, IQR 23-160 cp/capsule and peak of 955 cp/capsule. For RTdPCR, 55 of 107 (51%) RNA samples were positive for HEV. Measuring with RTdPCR, there was a median of 165 cp/capsule, IQR 92-395 cp/capsule and peak of 5800 cp/capsule. Pearson's correlation between RTqPCR and RTdPCR was modest with r=0.7729, P <0.0001 across 107 samples **(Supplemental Figure 3).** In total 64 of 107 samples were positive for HEV by either RTqPCR or RTdPCR. Thirty eight of 107 PERT capsules were positive for both RTqPCR and RTdPCR, whereas 9 of 107 samples were positive for only RTqPCR, and 17 of 107 samples were positive

for only RTdPCR. RTdPCR looks to out preform RTqPCR for these samples likely due to the low concentrations of target and high prevalence of inhibitors in samples. Digital PCR is thought to offer greater precision and copy number quantification due to it's binary nature.[18,19]

Whole Genome Sequencing

Despite efforts to perform amplicon based whole genome sequencing using long-range and over lapping HEV primers, we were unsuccessful in identifying HEV RNA by whole genome sequencing using PERT extracts. Three pooled PERT capsules from each of the four manufactures and the previous eight samples Sanger sequenced were unsuccessful with either method. Controls with positive patient HEV plasma (Case 2) were successful in generating overlapping PCR amplicons with had appropriate band sizes for all 8 amplicons spanning the HEV genome. No product was seen for PERT samples despite trying to adjust RNA, cDNA concentrations and adjusting cycle conditions. Bioanalysis at the UCalgary sequencing core facility of PERT extracted samples also indicated presence of inhibitors. To mitigate this, we attempted a second RNA clean up step, however, those samples were also unsuccessful. A review of the literature suggests the necessary template required for whole genome sequencing using serum, plasma or stool samples is 10^5 copies/ml,[11,13] whereas the highest PERT sample in our cohort did not exceed 10^3 copies/ml, suggesting limited template available for detection by WGS .

Protein Detection

Concentration experiments using 100kDa ultrafiltration of 10 PERT capsules to retain viral particles and filter out concentrated smaller proteins protease, lipase and amylase had variable

results, in part due to pH sensitive enteric coating. Enterically coated enzymes, even after significant centrifugation, precipitated and clogged the filter, leading to poor concentration and flow through of smaller size proteins which was observed across all samples (i.e., manufacture -1 concentrated 33-58%, manufacture-2 35-75%, manufacture-3 59-80% and manufacture-4 83-94%). SDS-page gels demonstrated the majority of proteins under 100kDa remained in the concentrated fraction instead of the intended filtrate. Despite the poor quality of the concentrate, Western blots for HEV ORF2 capsid protein were attempted but results inconculsive. Mass spectrometry was further attempted on subsequent samples without bands on Western Blots as original samples rapidly degraded. Results were of low yield for HEV proteins with only 3 protein matches with one peptide each for the positive tissue culture supernatant spiked sample.

Cell Culture

Both culture experiments produced positive supernatant RTqPCR results for the positive control using patient plasma (Case 2) in all the cell lines tried. However, tissue culture supernatant from PERT treated samples were negative by RTqPCR for each of the two methods described above. More robust purification away from enzyme matrix and concentration of virions to a minimum 10^4 cp/ml would be required in future expertiments.[17]

Given the presumed infrequency with which replication competent HEV is expected to exist in PERT, ongoing efforts to cultivate it from PERT were deemed to be underpowered for detection.

SUPPLEMENTAL FIGURES LEGEND

Supplemental Figure 1: Study flowchart and design of cohorts evaluated for HEV serostatus.

Supplemental Figure 2: Clinical course of the three CF lung transplant recipients with chronic HEV infection. The x-axis indicates number of days from first abnormal liver function testing. The left y-axis indicates the levels of liver function testing in either U/mL (AST, ALT, GGT, ALP) or μmol (Bilirubin total). The right y-axis indicates viral load (Altona Diagnostics RealStar HEV RT-PCR v2.0 assay, limit of detection of 0.20 IU/ul [95% CI: 0.12-0.45IU/ul]). Dotted black lines indicate the time of initial HEV serology positivity. Shaded regions indicate periods of time while cases were on treatment with ribavirin (RBV, light gray) or ribavirin and sofosbuvir (RBV + SOF, dark gray). Asterisks indicate that viral titres were not available during treatment period prior to becoming negative (serology was positive); thus, a mean viral titre was estimated and indicated by a dashed line for viral negativity (and to correspond with negative stool testing). AST: aspartate aminotransferase; ALT: alanine transaminase; GGT: gamma-glutamyl transferase; ALP: alkaline phosphatase; HEV: Hepatitis E Virus.

Supplemental Figure 3: Comparison of two different quantitative methods RTdPCR and RTqPCR for determining HEV RNA copies per PERT enzyme. Pearson correlation r=0.7732, R^2=0.5978, P =<0.0001, N=107

Supplementary Table 1: Primers and Probes Used for RTqPCR, RTdPCR and nested PCR

Primer Name	Sequence	HEV Gene Target (nucleotide*)	Reference
HEVH-3329 F1	AGCTCCTGTACCTGATGTTGACTC	PCR-2 *orf2* **(5622-5911)***	Huang, *et al.* 2002 [6]
HEVH-3330 R1	CTACAGAGCGCCAGCCTTGATTGC		
HEVH-3331 F2	GCTCACGTCATCTGTCGCTGCTGG		
HEVH-3332 R2	GGGCTGAACCAAAATCCTGACATC		
HEVB1 E3_12S	ACGYATGTGGTCGAWGCCATG	PCR-3 *orf1* **(22-561)*****	Munoz-Chimeno, *et al.* 2016 [7]
HEVB1 E3_987A	AARAGCATRAGCCGRTCCCA		
HEVB1 E3_22S	TCGAWGCCATGGAGGCCCA		
HEVB1 E3_561A	GTCATCCCRTGICGRGCCAT		
HEV_R4565	CCGGGTTCRCCIGAGTGTTTCTTCCA	PCR-1 *orf1* **(4228-4565)****	Drexler, *et al.* 2012 [5]
HEV_R4598	GCCATGTTCCAGAYGGTGTTCCA		
HEV_F4228	ACYTTYTGTGCYYTITTTGGTCCITGGTT		
HEV qPCR F	GGTGGTTTCTGGGGTGAC	*orf3* **(5311-5380)***	Salvio *et al.* 2018 [2]
HEV qPCR R	AGGGGTTGGTTGGATGAA		
HEVProbe FAM/BHQ1	TGATTCTCAGCCCTTCGC		
gBlock Modified with buffer bases	GGTACCGAGAACCTGTACTTCCAATCCAATGACTGATGCTCAGTGAGTTACTACGCAGTCACTCATAATACGACTCACTATAGTTCGTAGGGGTTGGTTGGATGAACGTAGCGAAGGGCTGAGAATCAATGCGTGTCACCCCAGAAACCACCTTCGTTATCTGGTGATACATGAACAGATCCGTGCACCGTCATTGGAAGTGGATAACGGATCCGAATTCGA		
BCoV F	CTGGAAGTTGGTGGAGTT	Exogenous spiked control	Decaro *et al*. 2008 [20]
BCoV R	ATTATCGGCCTAACATACATC		
BCoV probe FAM/IBHQ	CCTTCATATCTATACACATCAAGTTGTT		

*Sequence determined from GenBank accession number AF060669, ** GenBank accession number NC_001434, *** reference #7

Supplementary Table 2: Primers and Probes Used for HEV Whole Genome Sequencing

Primer Name	Sequence	HEV Amplification Site	Reference
HEV 1 HEV-15 f	TGTGGTCGAYGCCATGGAG	15-1129	Wang *et al*. 2018 [13]
HEV 1 HEV-23_r	CRTCCTCAGAGGCRTTCC		
HEV 2 HEV-24_f	GCTGYTCACGGCTWATGAC	1034-2108	
HEV 2 HEV-16_r	AAKGGATTGGCMGACTCCC		
HEV 3 HEV-137_f	TCTAATGGCCTGGACTGTACTG	1894-3176	
HEV 3 HEV-124_r	TGGACCGAYGAGGCYCGCTGCAT		
HEV 4 HEV-123_f	AGGGTTGAGCAGAACCCYAAGAGGC	2602-3831	
HEV 4 HEV-18_r	CTGYTCAAGCTCTGGGCARG		
HEV 5 HEV-157_f	TACCACCAGCTKGCTGAGGAG	3751-4622	
HEV 5 HEV-41_r	GCCATGTTCCAGACDGTRTTCCA		
HEV 6 HEV-28_f	ATGGAGGAGTGTGGBATGC	4465-5332	
HEV 6 HEV-20_r	GAAGGGGTTGGTTGGATG		
HEV 7 HEV-126_f	TGCCTATGCTGCCCGCGCCACC	5187-6325	
HEV 7 HEV-129_r	ACCYCCRGCCGACGAAATCAATTCTG		
HEV 8 HEV-05_f	CCGACAGAATTGATTTCGTCGG	6297-7123	
HEV 8 HEV-22_r	CTCCCGRGTTTTACCYACCT		
lrPCR F	AGGCCCAYCAGTTYATTAAGGCTCCTGGCATYACT	31	Papp *et al*. 2022 [11]
lrPCR R	CACACCCCTGCAAACCAAGRGCGCGRCACTCCGG	7,086	
Hemi-nested lrPCR R	CGGCACTCAGGGCAGAAATCATCRAAAGTRTGGG 7,063	7,063	
VN Primer	/5phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN	--	Nanopore protocol Direct cDNA Sequencing V14 with SQK-LSK114
Strand-switching Primer	TTTCTGTTGGTGCTGATATTGCTmGmGmG		
PR2 Primer	/5Phos/TTTCTGTTGGTGCTGATATTGC		

Supplementary Table 3: Pancreatic Enzyme Replacement Therapy Capsules Screened for HEV and Exogenous Spiked Control

PERT Capsule			BCoV			HEV			
ID	Manufacturer, Lot, Bottle, Capsule	Formulation Strength	Blank with Spike Cq	Spike Cq	1/10 Dilution Spike Cq	Cq	1/10 Dilution Cq	RTqPCR cp/capsule	RTdPCR Cp/capsule
PE05	2-29-I-A	Low	24.62	ND*	29.7	ND	38.14	207.52	592.50
PE06	1-15-I-A	High	24.98	ND*	ND	ND	ND	0	0.00
PE07	2-27-I-A	High	25.12	ND*	29.22	ND	ND	0.00	0.00
PE08	1-19-I-A	High	26.02	41.5*	31.92	ND	39.79	160.01	110.00
PE09	1-7-I-A	High	26.02	32.77*	29.26	ND	ND	0.00	226.67
PE10	4-38-I-A	High	25.12	ND*	ND	ND	ND	0.00	0.00
PE11	1-8-I-A	High	24.62	ND*	ND	ND	ND	0.00	0.00
PE12	2-30-I-A	Low	25.12	30.46*	29.13	ND	ND	0.00	0.00
PE13	1-20-I-A	High	25.12	42.49*	ND	ND	ND	0.00	0.00
PE14	1-2-I-A	Low	24.77	24.77	33.21	ND	ND	0.00	0.00
PE15	1-9-I-A	High	24.49	27*	29.68	42.06	43.22	20.57	275.00
PE16	1-10-I-A	High	24.34	27.86*	30.26	42.7	41.41	73.04	275.00
PE17	1-21-I-A	High	24.62	41.27	34.85	ND	40.29	125.68	792
PE18	4-39-I-A	High	24.62	36.94*	29.44	ND	ND	0.00	0.00
PE19	1-11-I-A	High	27.28	ND*	ND	ND	ND	0.00	0.00
PE20	1-12-I-A	High	24.62	ND*	ND	ND	ND	0.00	0.00
PE21	1-13-I-A	High	27.28	ND*	ND	ND	ND	0.00	0.00
PE22	1-16-I-A	High	25.12	39.91*	31.63	ND	40.51	108.50	460.00
PE22-2	2-26-IA	High	24.76	ND*	37.34	ND	ND	0.00	450.00
PE23	2-41-I-A	Low	24.76	38.32*	ND	ND	ND	0.00	0.00
PE24	1-42-I-A	Low	24.83	34.42*	35.9	ND	ND	0.00	0.00
PE25	1-14-I-A	High	26.02	28.49*	29.11	44	40.54	62.13	73.33
PE27	1-4-II-A	High	24.69	34.18*	ND	ND	ND	0.00	165.00
PE28	1-4-II-B	High	24.89	34.21*	ND	ND	ND	0.00	165.00
PE29	1-4-II-C	High	26.02	ND*	ND	ND	ND	0.00	0.00

PE31	1-4-I-A	High	24.96	29.53*	28.99	ND	ND	0.00	137.50
PE32	1-4-I-B	High	25.22	28.4*	29.91	ND	ND	0.00	137.50
PE33	2-24-I-A	High	25.22	26.29	28.77	38.38	37.64	476.82	4237.50
PE35	1-4-I-C	High	25.08	26.34	28.79	44.66	ND	0.00	137.50
PE36	2-24-I-B	High	25.08	26.78	28.77	39.4	38.04	363.52	5800.00
PE37	2-24-I-C	High	24.15	25.73	28.04	38.08	38.14	535.83	4870.00
PE38-39	2-24-I-DE	High	24.15	27.64*	26.95	42.76	36.16	955.32	3000.00
PE40	1-3-I-A	High	26.02	35.91*	29.59	ND	ND	0.00	91.67
PE41	1-4-II-C	High	26.02	33.51*	29.38	ND	ND	0.00	0.00
PE42	4-36-I-A	High	24.84	ND*	30.5	ND	ND	0.00	0.00
PE43	4-36-I-B	High	26.02	36.53*	27.84	ND	ND	0.00	0.00
PE44	2-40-I-D	Low	24.87	30.22*	28.75	ND	ND	0.00	0.00
PE45	2-40-I-E	Low	24.84	38.66*	36.38	ND	ND	0.00	0.00
PE46	2-40-I-F	Low	26.02	29.28*	29.08	ND	ND	0.00	0.00
PE47	4-36-I-C	High	24.87	ND*	32.21	ND	ND	0.00	0.00
PE48-49	1-4-II-DE	High	25.92	38.16*	29.48	ND	ND	0.00	27.50
PE50-51	1-3-I-BC	High	27.02	25.92	30.9	ND	40.15	14.95	197.50
PE52-53	1-4-I-DE	High	30.44	25.92	30.34	ND	41.89	4.57	27.50
PE54	4-36-II-A	High	24.83	30.5*	30.19	ND	ND	0.00	0.00
PE55	4-36-II-B	High	24.83	32.45*	30.29	ND	ND	0.00	170.00
PE56	2-24-I-F	High	23.86	25.99*	28.6	35.74	36.49	422.16	2490.00
PE61	1-4-I-F	High	32.417	28.147	41.21	40.34	38.6	477.00	0.00
PE67	2-25-I-A	High	26.95	36.38*	38.91	ND	38.14	428.25	1360.00
PE68	2-25-I-B	High	26.95	35.6*	39.02	38.43	38.11	585.72	3420.00
PE69	2-22-I-A	High	24.57	30.46*	28.42	ND	41.27	27.54	99.00
PE70	2-22-I-B	High	24.57	35.55*	28.91	ND	ND	0.00	0.00
PE71	2-22-I-C	High	24.57	34.64*	28.85	ND	ND	0.00	110.00
PE72	2-22-I-D	High	24.57	29.9*	28.54	ND	ND	0.00	0.00
PE73	2-22-I-E	High	24.57	31.2*	28.44	ND	ND	0.00	110.00
PE74	1-5-I-A	High	30.56	27.06	28.51	ND	ND	0.00	230.00
PE75	1-5-I-B	High	30.56	31.93	28.84	ND	41.19	34.89	110.00
PE76	1-5-I-C	High	30.56	25.59	28.12	ND	41.85	22.51	0.00

PE77	1-5-I-D	High	30.56	26.84	28.74	44.74	40.67	49.67	340.00
PE78	1-5-I-E	High	30.56	27.06	28.76	ND	ND	0.00	0.00
PE79	3-34-I-A	Low	24.83	24.38	27.1	41.72	ND	1.23	55.00
PE80	3-34-I-B	Low	24.83	27.92*	27.91	ND	ND	0.00	0.00
PE81	3-34-I-C	Low	24.83	25.47	27.25	ND	ND	0.00	0.00
PE82	3-34-I-D	Low	24.83	24.77	27.33	44.83	ND	0.00	55.00
PE83	3-34-I-E	Low	24.83	25.13	27.33	ND	ND	0.00	0.00
PE84	3-31-I-A	Low	24.83	29.15*	28.01	ND	ND	0.00	0.00
PE85	3-31-I-B	Low	24.83	25.11	27.35	ND	41.07	30.42	115.00
PE86	3-31-I-C	Low	24.83	26.28	27.23	ND	ND	0.00	170.00
PE87	3-31-I-D	Low	24.83	25.37	27.37	ND	41.77	24.98	55.00
PE88	3-31-1-E	Low	24.83	26.61	27.3	ND	39.49	83.44	55.00
PE89	3-32-I-A	High	25.13	33.57*	30.42	ND	ND	0.00	0.00
PE90	3-32-I-B	High	25.13	26.6	30.03	ND	40.84	42.41	0.00
PE91	3-32-I-C	High	25.13	26.35	29.09	40.07	41.84	19.60	0.00
PE92	3-32-I-D	High	25.13	26.12	29.55	40.1	39.14	123.98	0.00
PE93	3-32-I-E	High	25.13	28.55*	29.31	44.86	40.02	66.26	110.00
PE94	2-28-I-A	Low	30.56	26.58	29.24	39.02	40.87	18.41	55.00
PE95	2-28-I-B	Low	30.56	27.87	28.64	ND	39.49	53.99	55.00
PE96	2-28-I-C	Low	30.56	28.85	29.72	ND	41.64	10.75	110.00
PE97	2-28-I-D	Low	30.56	26.11	27.27	38.41	37.3	329.82	285.00
PE98	2-28-I-E	Low	30.56	25.56	27.56	ND	36.97	275.89	735.00
PE99	2-23-I-E	High	25.13	29.24*	28.74	ND	39.82	86.64	230.00
PE100	2-23-I-A	High	25.13	30.54*	29.06	ND	41.48	2.21	46.00
PE101	2-23-I-B	High	25.13	32.87*	28.87	ND	40.72	40.73	230.00
PE102	2-23-I-C	High	25.13	31.53*	28.77	ND	40.91	56.21	110.00
PE103	2-23-I-D	High	25.13	31.04*	29.35	ND	40.4	50.23	230.00
PE104	1-1-I-A	Low	24.83	28.95*	28.63	ND	ND	0.00	0.00
PE105	1-1-I-B	Low	24.83	28.94*	28.54	ND	ND	0.00	0.00
PE106	1-1-I-C	Low	24.83	32.64*	29.59	ND	ND	0.00	0.00
PE107	1-1-I-D	Low	24.83	28.77*	28.51	ND	ND	0.00	0.00
PE108	1-1-I-E	Low	24.83	27.42*	28.49	ND	ND	0.00	0.00

PE109	3-32-II-A	High	23.86	25.56	29.01	38.9	41.93	10.00	55.00
PE110	3-32-II-B	High	23.86	27.09*	30.08	39.91	39.99	38.45	395.00
PE111	3-32-II-C	Low	23.86	28.44*	30.82	ND	ND	0.00	0.00
PE112	3-32-II-D	High	23.86	26.71*	30.01	40.21	ND	3.26	11.00
PE113	3-32-II-E	High	23.86	28.19*	30.23	42.37	40.53	26.26	11.00
PE114	2-40-I-A	Low	24.83	32.92*	28.69	ND	ND	0.00	565.00
PE115	2-40-I-B	Low	24.83	34.28*	34.51	ND	ND	0.00	0.00
PE116	2-40-I-C	Low	24.78	36.91*	36.03	ND	ND	0.00	0.00
PE117	4-35-I-A	Low	24.78	29.18*	30.86	ND	ND	0.00	0.00
PE118	4-35-I-B	Low	24.78	29.94*	31.01	ND	ND	0.00	0.00
PE119	4-35-I-C	Low	24.78	30.21*	30.67	ND	ND	0.00	0.00
PE120	4-35-I-D	Low	24.78	30.77*	30.91	ND	ND	0.00	0.00
PE121	4-35-I-E	Low	24.78	29.15*	31.39	41.89	41.35	22.35	0.00
PE122	4-37-I-A	High	24.78	27.16*	29.54	ND	39.68	47.06	340.00
PE123	4-37-I-B	High	24.78	36.82*	31.68	ND	40.89	20.45	0.00
PE124	4-37-I-C	High	24.78	34.04*	32.91	ND	40.08	35.65	0.00
PE125	4-37-I-D	High	24.78	44.03*	33.62	ND	40.75	22.53	0.00
PE126	4-37-I-E	High	24.78	ND*	32.2	ND	ND	0.00	0.00

Canadian PERT Manufactures are coded as 1-4.
PERT capsule strength are coded as low ≤10,000 units of lipase and high >10,000 units of lipase.
ND = Not determined, Cq= Cycle of Quantification.
*Indicates presence of inhibitors as noted by an increase Cq >2 for spiked sample compared to spiked buffer.

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