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Original research

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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ABSTRACT

Objectives In high-income countries hepatitis E virus (HEV) is an uncommonly diagnosed porcine-derived zoonoses. After identifying disproportionate chronic HEV infections in persons with cystic fibrosis (pwCF) postlung transplant, we sought to understand its epidemiology and potential drivers.

Design All pwCF post-transplant attending our regional CF centre were screened for HEV. HEV prevalence was compared against non-transplanted pwCF and with all persons screened for suspected HEV infection from 2016 to 2022 in Alberta, Canada. Those with chronic HEV infection underwent genomic sequencing and phylogenetic analysis. Owing to their swine derivation, independently sourced pancreatic enzyme replacement therapy (PERT) capsules were screened for HEV.

Results HEV seropositivity was similar between transplanted and non-transplanted pwCF (6/29 (21%) vs 16/83 (19%); p=0.89). Relative to all other Albertans investigated for HEV as a cause of hepatitis (n=115/1079, 10.7%), pwCF had a twofold higher seropositivity relative risk and this was four times higher than the Canadian average. Only three chronic HEV infection cases were identified in all of Alberta, all in CF lung transplant recipients (n=3/29, 10.3%). Phylogenetics confirmed cases were unrelated porcine-derived HEV genotype 3a. Ninety-one per cent of pwCF were taking PERT (median 8760 capsules/person/year). HEV RNA was detected by RT-qPCR in 44% (47/107) of PERT capsules, and sequences clustered with chronic HEV cases.

Conclusion PwCF had disproportionate rates of HEV seropositivity, regardless of transplant status. Chronic HEV infection was evident only in CF transplant recipients. HEV may represent a significant risk for pwCF, particularly post-transplant. Studies to assess HEV incidence and prevalence in pwCF, and potential role of PERT are required.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Hepatitis E virus (HEV) is a porcine-derived zoonoses infrequently identified as a cause of hepatitis in high-income countries.
⇒ In Europe and North America, exposure has been epidemiologically linked to the consumption of pork products, although direct identification of HEV RNA in meat products marketed to humans is exceptionally uncommon.
⇒ Among heavily immunosuppressed populations, HEV can cause a chronic hepatitis (and a range of extrahepatic manifestations) that ultimately may progress to fulminant liver failure.

WHAT THIS STUDY ADDS

⇒ Chronic HEV infections are exceptionally rare, and this is the first study to report infection in persons with cystic fibrosis (pwCF) (three separate, unrelated cases in a single regional centre).
⇒ We observed rates of HEV seropositivity to be similar among pwCF, regardless of lung transplant status, and these rates were four times higher than the published Canadian national average, and even twice that of all individuals in the Province of Alberta specifically referred for HEV testing (ie, a preselected, at-risk group).
⇒ We hypothesised porcine-derived pancreatic enzyme replacement therapy (PERT), a medication taken by ~90% of individuals with CF on account of pancreatic insufficiency (median ~24 capsules/day), may represent a biologically plausible source of infection to explain HEV disproportionate occurrence in pwCF.
⇒ We found HEV RNA in 44% of PERT capsules, including all formulations from all Canadian manufacturers; moreover, PERT HEV *orf1* gene sequence clustered with both CF-associated HEV infection cases and Canadian swine herds—suggesting a potential iatrogenic mechanism of infection.

INTRODUCTION

Hepatitis E virus (HEV) is a leading cause of viral hepatitis worldwide, particularly in low-income and middle-income countries.[1] HEV is a non-enveloped, positive-sense, single-stranded RNA virus of 7.2 kb with seven genotypes; however, only genotypes 1–4 (and rarely 7) display human tropism. The WHO estimates 20 million incident infections globally, with 3.3 million symptomatic and an associated 50 000 deaths. In high-income countries, genotypes

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

- ⇒ The high frequency of comorbid CF liver disease (one-third of individuals) may limit investigations into alternate liver disease aetiologies such as HEV, limiting its identification in other CF cohorts.
- ⇒ This study should prompt others to explore the prevalence of HEV (seropositivity and chronic infection) in other CF, and other PERT using cohorts.
- ⇒ Similarly, this study warrants a re-analysis of PERT safety as an agent with potential for zoonotic infection risk—especially in profoundly immunosuppressed population, such as CF lung transplant recipients.

3 and 4 are endemic in domestic swine herds and wild game, with rare sporadic zoonotic hepatitis cases reported in humans. Predominately an agent causing hepatitis, extrahepatic manifestations can occur and include neurological syndromes, renal injury and pancreatitis. Historically, HEV was considered an acute self-limited infection. However, in profoundly immunocompromised populations, chronic infections associated with genotype 3 are increasingly described.[1 2] These infections are autochthonous and thought to be acquired from direct contact with or from ingestion of undercooked pork or wild game products.[3] In solid organ transplant (SOT) recipients with acute HEV infection, as many as two-thirds progress to chronic hepatitis with sequalae including cirrhosis and death.[1]

Cystic fibrosis (CF), a multisystem disorder, is the most common fatal genetic disease among Caucasians. As approximately 20% of adult persons with CF (pwCF) living in Canada are lung transplant recipients,[4] understanding factors impacting this high-risk population is critical. After identifying three cases of chronic HEV infection in our CF regional centre, these being only the second, third and fourth cases of chronic HEV infection ever described in Canada, we sought to assess the prevalence and clinical outcomes across a cohort of transplanted and non-transplanted pwCF. We hypothesised that this apparent cluster of HEV chronic disease observed in pwCF was the result of disproportionate HEV exposure risk and sought to determine seroprevalence of HEV among pwCF and determine if this was increased relative to non-CF populations. As pancreatic insufficiency (PI) is among the most prominent phenotypes of CF, with afflicted individuals requiring high doses of porcine-derived pancreatic enzyme replacement therapy (PERT), we hypothesised PERT represented an HEV zoonotic exposure risk specific to pwCF and sought to determine if HEV RNA contaminated the PERT consumed by pwCF. An overview of the study rationale and results are detailed in the graphical abstract (figure 1).

METHODS

Study design and selection of subjects

Three cohorts were included in the study (online supplemental figure 1):

1. *CF-SOT recipients*. After clinical cases of chronic HEV infection were identified in the Southern Alberta Adult CF Clinic, we initiated anti-HEV IgG and IgM serological, and HEV RNA screening (as positive cases can occur in the absence of seropositivity[5]) in all CF transplant recipients.
2. *A prospectively enrolled CF cohort* that had not previously received a SOT from the same regional CF centre.
3. *All individuals in the Province of Alberta (total population of ~4.5 million) with clinical suspicion for HEV as a potential cause of liver disease* (ie, those with elevated liver enzymes in which other causes of hepatitis were ruled out) for whom HEV testing was performed between January 2016 and August 2022 (ie, a preselected, at-risk group).

Among pwCF, demographics and treatments at time of HEV testing were recorded. PwCF with PI were identified based on clinician prescribed PERT. PERT use was classified by manufacturer, formulation and total number of capsules taken per day.

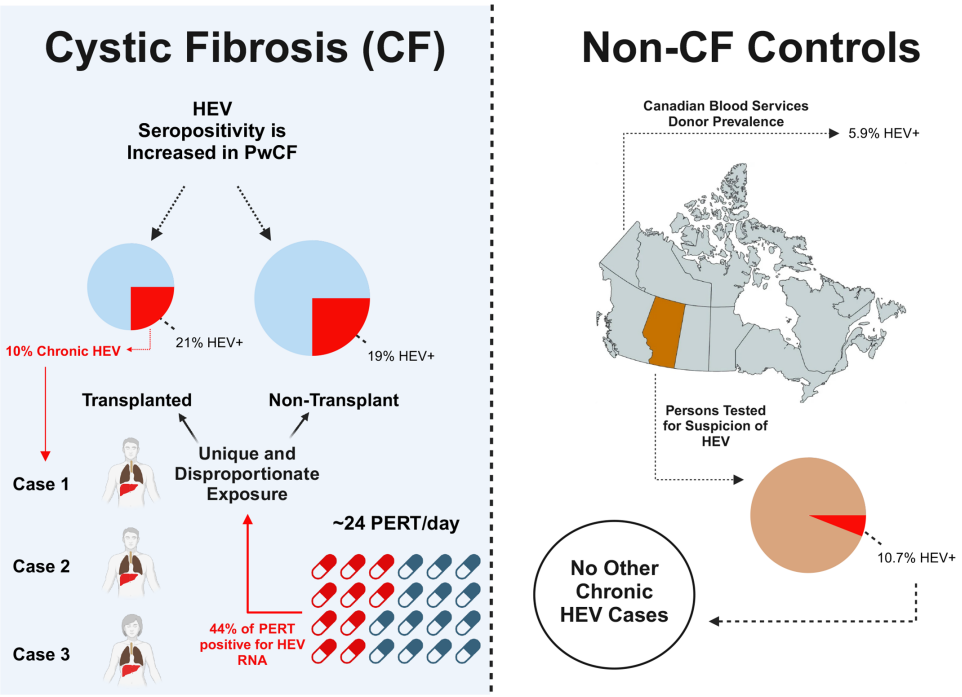

Figure 1 Graphical abstract. HEV, hepatitis E virus; PERT, pancreatic enzyme replacement therapy; pwCF, patients with cystic fibrosis.

For the remaining subjects, age, sex, transplant status and immunosuppressive regimen were recorded.

Clinical HEV serology and RNA testing

Serum and plasma from all transplant recipients (and stool for those under suspicion) were assessed at the National Microbiology Laboratory (Winnipeg, Manitoba, Canada) for clinical care purposes. HEV IgG and IgM were assessed using ELISA (Wantai Biopharm, Beijing, China). HEV IgG assessed for research purposes in the non-transplanted CF cohort was performed using two complementary assays (Abbexa Ca. ABX364866 and Elabscience Biotechnology Ca. E-HD-E055). Participants were considered HEV seropositive only when both IgG assays were positive. Additional details on the method for these measurements are provided in the online supplemental file.

Plasma samples (250 µL) received at the National Microbiology Laboratory for HEV RNA detection were extracted by silica-coated magnetic bead purification using the NUCLISENS EASYMAG instrument (Biomérieux Canada, Saint-Laurent, Quebec, Canada). In the research laboratory, nucleic acid was extracted from plasma using QIAamp MinElute Virus spin kit (Ca. 57705, CPN 1198016.6) with a modified protocol using 400 µL plasma and increasing kit reagent volumes by 2× and using carrier RNA as described by the instructions from the manufacturer.

Pancreatic enzyme detection of HEV

PERT was independently acquired from pwCF and pharmacies to ensure broad representation from Southern Alberta and a diverse range of formulations and lots. Eleven formulations of PERT capsules produced by four manufacturers (all manufacturers with Health Canada-approved products) were screened and lot variability determined with replicate capsules. Dissolved PERT capsules first underwent TRIzol (Thermo Fisher Scientific) extraction owing to the extreme protein-rich extract and the confounding effects of (most) PERT's enteric coating followed by cleanup using QIAamp MinElute Virus spin columns. Assessment for molecular and genomic inhibitors in PERT extracts was assessed (see online supplemental file for details). The presence of HEV RNA was assessed using an *orf3* RT-qPCR and with an *orf3* RT-digital PCR (RT-dPCR) each with appropriate positive and negative controls (online supplemental table 1). Nested PCR and Sanger sequencing was performed on a subset of select HEV RNA-positive PERT samples targeting two regions of HEV *orf1* (both the 5' end and 3' end) and *orf2* (figure 2, online supplemental tables 1 and 2). HEV sequences identified from pwCF and PERT from this study are uploaded into GenBank (SUB14282691).

HEV RNA genotyping

Phylogenetic analysis of output and reference sequences was performed by maximum likelihood inference of a 315 bp trimmed *orf1* alignment using DIVEIN web tools by the TN93+γ+I model (online supplemental material).

Statistical analyses

Descriptive statistics were completed in comparing cohorts. Differences between groups were assessed using the Wilcoxon rank-sum test or Fisher's exact test, with $p<0.05$ considered statistically significant. Chi-squared test was used to compare discrete variables between groups. Relative risk (RR) for HEV was calculated between cohorts (ie, those transplanted vs non-transplanted and pwCF vs non-CF controls). Statistical analysis was performed using R, V.4.04 (R Core Team, 2021).

RESULTS

Prevalence of HEV seropositivity and clinical disease among CF transplant recipients

After identifying the first three cases of HEV clinical disease ever reported in Alberta, Canada, all among pwCF attending the Southern Alberta Adult CF Clinic, we screened all transplanted pwCF attending the clinic for HEV seropositivity (IgG and IgM) and HEV RNA as part of an evolved clinical care pathway. Across the cohort of transplanted pwCF (n=29), all of whom had previously received life-saving lung transplants, six subjects were HEV IgG seropositive (20.7%) (table 1). Three of these individuals were also positive for HEV RNA (10.3%) and these comprised the chronic HEV infection cohort (table 2).

Transplanted pwCF that were determined to be HEV seropositive were younger at the time of transplant (median (IQR) 28.6 years (22.1–32.5)) as compared with HEV seronegative (31.3 years (25.6–36.1); p=0.037) (table 1). The duration of time from transplant surgery was associated with HEV seropositivity, whereas individual age was not. Those who were >3 years post-transplant had an RR of 2.53 for HEV seropositivity (1.48–4.23, p=0.023) compared with those <3 years post-transplantation. The type and amounts of transfused blood products (eg, whole blood, platelets, plasma) did not differ between groups at the time of transplantation, and no HEV seropositive individual ever received any additional blood product after the original lung transplant surgery. We determined HEV serostatus did not associate with the amount of PERT consumed among transplanted pwCF (22 PERT capsules/day (IQR 18–26) among HEV seropositive individuals vs 23 PERT capsules/day among HEV seronegative (IQR 19–26); p=0.86). Furthermore, other characteristics of transplanted pwCF, including age and sex, did not differ by HEV serostatus (table 1).

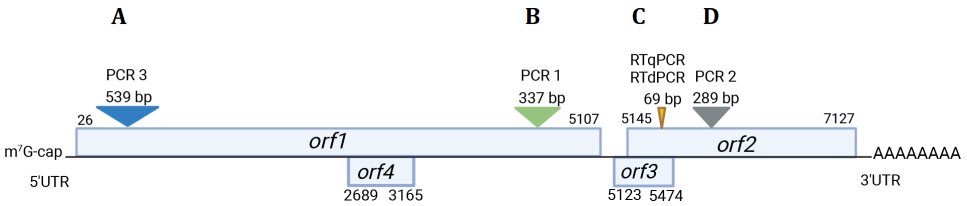

Figure 2 HEV genome and the molecular and genomic targets used in this study. Of the 7.2 kb genome, four independent areas were identified including (A; PCR-3) a nested PCR yielding a 539 bp sequenced target at the 5′ end of *orf1*; (B; PCR-1) a nested PCR yielding a 337 bp sequenced product in *orf1* at the 3′ end; (C) quantitative and digital-PCR—product of 69 bp in size from *orf2/3* and (D; PCR-2) a nested PCR yielding a 289 bp sequenced product in *orf2*. UTR, untranslated region.

Table 1 Demographics of adult CF lung transplant recipients and HEV serostatus

	HEV seropositive (n=6), N (%)	HEV seronegative (n=23), N (%)
Characteristic		
Female sex	2 (33)	13 (57)
Median age, years (IQR)	40.4 (34.1–47.3)	37.6 (29.3–49.5)
Rural postal code	1 (17)	5 (22)
CF-related factors		
Genotype		
ΔF508 homozygous	4 (67)	14 (61)
ΔF508 heterozygous	2 (33)	8 (35)
Other	0	1 (4)
CF comorbidities		
Pancreatic insufficiency	6 (100)	20 (87)
CF liver disease	3 (50)	7 (30)
GORD	3 (50)	16 (70)
Distal intestinal obstruction syndrome	6 (100)	21 (91)
CF arthropathy	1 (17)	2 (9)
Sinus disease (polyps or nasal congestion)	6 (100)	19 (83)
Pretransplant factors		
Median age at time of transplant, years (IQR)	28.6 (22.1–32.5)	31.3 (25.6–36.1)
Median BMI at time of transplant (IQR, kg/m^2)	20.9 (18.7–22.1)	19.9 (18.4–21.9)
Per cent predicted forced expiratory volume predicted at time of transplant, median (IQR)	22.7 (15.1–28.3)	23.5 (16.0–31.8)
CF-related diabetes (pretransplant prevalence)	3 (50)	11 (48)
Chronic *Pseudomonas aeruginosa* infection	6 (100)	21 (91)
Post-transplant factors		
CF-related diabetes (post-transplant prevalence)	4 (67)	15 (66)
Blood product received at time of transplant	6 (100)	20 (87)*
CMV status		
Donor+, recipient–	2 (33)	7 (30)
Donor+, recipient+	4 (67)	15 (66)
Other	0	1 (4)
Maintenance immunosuppression†		
Tacrolimus	6 (100)	22 (96)
Corticosteroids	6 (100)	23 (100)
Mycophenolate	5 (83)	20 (87)
Azathioprine	1 (17)	3 (13)
Sirolimus	0	1 (4)
Patients with ≥1 acute cellular rejection episode	2 (33)	9 (29)

*Reported for those with available records at time of transplantation. Two individuals in the seronegative group did not have records available.
†Patients were often on multiple drug regimens and thus numbers greater than total sum of patients. Immunosuppressive regimen at time of HEV serostatus determination is reported.
BMI, body mass index; CF, cystic fibrosis; CMV, cytomegalovirus; HEV, hepatitis E virus.

Chronic HEV infections in transplanted pwCF

Between March 2017 and December 2022, three chronically infected HEV individuals were identified in our transplanted CF cohort (figure 3, table 2 and online supplemental figure 2).

Case 1 was a man with CF aged 40 years with no history of CF liver disease (CFLD) 2 years post-transplant when liver function abnormalities were first noted. HEV serology (IgG and IgM) and RT-qPCR were eventually found to be positive, and he was initiated on ribavirin at 600 mg (~8 mg/kg/day). He developed drug toxicity despite mitigating efforts and his viraemia was not resolved at any point through 2.5 years of therapy, leading to treatment interruption and observation. Four years following the initial HEV identification, he developed a progressive neurological syndrome including persistent nausea and weight loss and eventually gait instability and cognitive impairment. The patient was again initiated on ribavirin with subsequent addition of sofosbuvir. MRI of the brain demonstrated white matter changes and cerebrospinal fluid (CSF) analysis demonstrated a lymphocytic pleocytosis (white blood cell count 12.6×10^6/L, 97% lymphocytes) and HEV RNA presence was confirmed. Investigations for all other aetiologies to explain functional and cognitive decline were negative. The patient was diagnosed with HEV encephalopathy. Despite treatment, the patient's neurological status progressively deteriorated, and he passed away 9 months later, ~6.5 years after HEV diagnosis. The family declined an autopsy.

Case 2 was a man with CF aged 41 years postlung transplantation with no history of CFLD. He developed abnormal liver enzymes 8 years after transplant and was ultimately found to be HEV IgG/IgM and RNA positive. He received ribavirin 600 mg (~8 mg/kg/day) and had documented HEV clearance (plasma and stool) after 10 months of therapy. One year later, transaminitis recurred and repeat testing demonstrated HEV RNA in plasma confirming recurrence/relapse. He was re-initiated on ribavirin but, despite mitigating efforts, was intolerant of treatment-related nausea and fatigue. This, combined with recalcitrant viral load at 9 months, led to discontinuation and observant management. Five years postdiagnosis, the patient continues to have chronic HEV infection with increasing transaminases, but unchanging liver stiffness (6.3 kPa by FibroScan, Echosens) and unwilling to consider retreatment. Because of nausea and weight loss and mood changes, neurological investigations were undertaken which did not show any MRI white matter changes and an LP did not reveal evidence of HEV RNA or lymphocytic pleocytosis.

Case 3 was a woman aged 36 years 9 years postliving-related lung transplantation. She developed abnormal liver enzymes and HEV IgM and RNA were identified. She was initiated on ribavirin therapy at 600 mg (~8 mg/kg/day) for 12 months but by 8 months was already PCR negative in plasma and stool. Repeat testing the next year demonstrated recurrence of HEV RNA, despite normal liver enzyme tests. Due to treatment-related side effects, a 2-year hiatus from repeat treatment with frequent follow-up is planned.

HEV seropositivity among non-transplanted pwCF

Given the high prevalence of HEV seropositivity in our transplanted CF cohort, we sought to explore HEV seroprevalence among a non-transplanted CF control cohort. Patients attending the same clinic were approached to participate in a prospective research protocol. Eighty-three pwCF volunteered, and 16 (19.3%) were positive for HEV IgG using two independent assays. None were positive for HEV RNA. Demographics of the control non-transplanted CF cohort are presented in table 3. No demographic or clinical factor, including patient age were found to be associated with increased risk of HEV seropositivity in this cohort. We did not observe a higher likelihood of HEV seropositivity in CF transplant recipients relative to those who had not received a transplant (RR 1.07, 95% CI 0.46 to 2.48; p=0.87).

Consistent with other CF cohorts, ~90% of individuals in each of the transplanted and non-transplanted CF cohorts were pancreatic insufficient. Furthermore, total number of PERT capsules consumed/day did not differ on the basis of transplant status (23 (IQR 19–26) vs 24 (IQR 20–27); p=0.79). Finally, PERT capsules consumed/day among non-transplanted pwCF

Table 2 Demographic features of CF transplant recipients with chronic HEV infection

	Subject 1	Subject 2	Subject 3
Age (years) at time of HEV detection	40	41	36
Biological sex	Male	Male	Female
CFTR mutation	F508del/F508del	F508del/F508del	F508del/F508del
Organ transplanted	Lung	Lung	Lung
Months since transplantation at time of HEV detection	24	91	111
Induction therapy	Rabbit antithymocyte globulins	Rabbit antithymocyte globulins	Rabbit antithymocyte globulins
Immunosuppressive therapy	Tacrolimus trough target 8–10 µg/mL/ mycophenolate mofetil 1 g two times per day/prednisone 5 mg	Tacrolimus trough target 8–10 µg/mL/ mycophenolate mofetil 1 g two times per day/prednisone 5 mg	Tacrolimus trough target 8–10 µg/mL/ mycophenolate mofetil 1 g two times per day/ prednisone 5 mg
Blood product receipt	Packed red blood cells and irradiated platelets at time of lung transplantation. None after transplant	Packed red blood cells and irradiated platelets at time of lung transplantation. None after transplant	Packed red blood cells and irradiated platelets at time of lung transplantation. None after transplant
CF-related comorbidities	Pancreatic insufficiency, *Pseudomonas aeruginosa* colonisation, DIOS, CF-related diabetes	Pancreatic insufficiency, *Pseudomonas aeruginosa* colonisation, DIOS	Pancreatic insufficiency, *Pseudomonas aeruginosa* colonisation, chronic rhinosinusitis, CF-related diabetes
Additional post-transplant-related comorbidities	Chronic kidney disease	Diabetes mellitus, chronic kidney disease	Chronic lung allograft dysfunction
Outcome	Chronic HEV encephalopathy and death	Observation due to ongoing HEV IgM+ and HEV RT-PCR+, intolerance to therapy, increasing transaminases and declining renal function	Observation due to recurrence post-treatment and reluctance to restart therapy owing to intolerance

CF, cystic fibrosis; DIOS, distal intestinal obstruction syndrome; HEV, hepatitis E virus.

did not differ on the basis of HEV serostatus (23 (IQR 20–27) vs 24 (IQR 22–26); p=0.63).

HEV seroprevalence in non-CF populations

Next, we sought to discern the seroprevalence of HEV in a non-CF control population using the only other dataset available—those referred for HEV testing based on clinical suspicion (ie, a preselected, at-risk population). In total, 1079 HEV serology tests were requested in all of Alberta during this 7-year period (total population of ~4.5 million residents), which was a small proportion (0.06%) of total viral hepatitides testing (hepatitis B surface antigen; total of 345 180 tests and antihepatitis C antibody; total of 1 406 224) completed by the provincial laboratory (note: much of the HBV and HCV (but not HEV) testing is also performed in regional laboratories and therefore under-reported using only the provincial health laboratory data). One hundred and fifteen Alberta residents who did not have CF (10.7%) were seropositive for HEV IgG. Thirteen (11.3%) were SOT recipients with the most prevalent being liver (n=8) followed by kidney (n=5). Despite the Southern Alberta Adult CF Clinic comprising only 0.0056% of Alberta's population, all cases of chronic HEV hepatitis occurred in pwCF and there

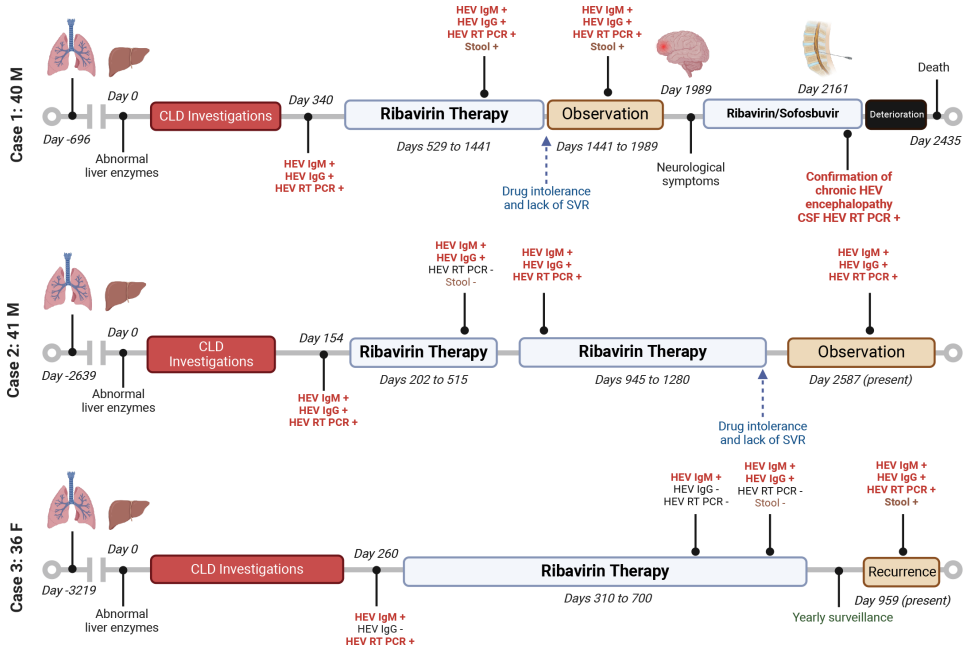

Figure 3 Timeline of transplanted subjects with CF with chronic HEV. Abnormal serology and viral RNA are indicated in red font. CF, cystic fibrosis; CLD, chronic liver disease; CSF, cerebrospinal fluid; SVR, sustained virological response.

Table 3 Demographics of adults persons with CF without prior transplant and HEV serostatus

	HEV seropositive (n=16) N (%)	HEV seronegative (n=67) N (%)
Characteristic		
Female sex	8 (50)	28 (42)
Median age, years (IQR)	30.9 (24.9–37.5)	32.0 (25.0–39.3)
Median body mass index, median (IQR, kg/m^2)	23.2 (22.0–24.1)	23.3 (20.9–25.1)
Rural postal code	5 (31)	22 (33)
Lung disease stage**		
Mild	8 (50)	36 (54)
Moderate	5 (31)	21 (31)
Severe	3 (19)	10 (15)
Genotype		
ΔF508 homozygous	10 (63)	40 (60)
ΔF508 heterozygous	5 (31)	21 (31)
Other	1 (6)	6 (9)
CF comorbidities		
Pancreatic insufficiency	16 (100)	60 (90)
CF liver disease	3 (19)	16 (24)
GORD	9 (56)	41 (61)
Distal intestinal obstruction syndrome	14 (88)	42 (63)
CF arthropathy	3 (19)	9 (13)
Sinus disease (polyps or nasal congestion)	15 (94)	55 (82)
Respiratory infections		
Pseudomonas aeruginosa	9 (56)	39 (58)
Methicillin-susceptible *Staphylococcus aureus*	6 (39)	27 (40)
Methicillin-resistant *S. aureus*	1 (6)	5 (8)
Other†	4 (25)	15 (22)

*Definitions of lung stage based on forced expiratory volume in 1 s; mild ≥70%, moderate ≥40% to <70%, severe <40%.
†Other bacterial species include *Escherichia coli, Enterobacter cloacae, Serratia marcescens,* group C *Streptococcus, Stenotrophomonas maltophilia*.
CF, cystic fibrosis; HEV, hepatitis E virus.

were no additional cases of HEV RNA positivity documented in the entirety of Alberta. Furthermore, PwCF, regardless of clinical suspicion or transplant status, had an increased HEV seropositivity risk relative to those individuals in Alberta who were specifically referred for HEV testing (22/112 (19.6%) vs 115/1079 (10.7%), RR 1.84 (95% CI 1.22 to 2.78), p=0.004).

HEV detection in pancreatic enzyme replacement therapy capsules

HEV RNA was detected by *orf2* RT-qPCR in 47/107 (44%) of PERT capsules assessed based on a RT-qPCR and confirmed with RT-dPCR (figure 4, online supplemental table 3, online supplemental figure 3). Positive PERT had a median value of 50 HEV copies/capsule, IQR 23–160 copies/capsule and peak of 955 copies/capsule, by RT-qPCR. When serial samples from within the same lot were assessed by RT-qPCR, 8/16 (50%) demonstrated HEV RNA concordance among all capsules, and 50% of lots had capsules that were both positive and negative. HEV RNA was detected in PERT from all four Health Canada-approved manufacturers, although rates differed slightly (10/37 (27%), 19/33 (58%), 11/20 (55%) and 5/17 (29%), p=0.027). Three separate nested-RT-PCR assays followed by Sanger sequencing confirmed HEV RNA in qPCR-positive PERT in *orf1* (including both the 5' and tailing portion of the gene) and *orf2* (figure 2). Efforts to identify HEV from PERT by whole genome sequencing, cell culture and protein assays were unsuccessful (see online supplemental file for details).

Phylogeny of HEV RNA in transplanted pwCF and PERT

The maximum likelihood phylogenetic tree of the three pwCF with chronic HEV infection and eight HEV-positive PERT was completed based on all available clinical samples in the National Microbiology Laboratory database (figure 4). The phylogenetic relationships among the different clades were confirmed by bootstrap values >70%. Based on phylogenetic analysis, the three postlung transplanted pwCF were infected with different HEV genotype 3a strains, confirming their independent acquisition. CF-associated chronic HEV cases and PERT sequences clustered with Canadian swine strains and other Canadian human HEV sequences. Notably, in cases 2 and 3 which demonstrated recurrence/relapse after apparent cure, across the 315 bp assessed there were 4 and 2 single nucleotide polymorphisms (2 and 0 differing amino acids) between the first and final isolates, respectively. As substitution rates in *orf1* are expected to be <1/year, these potentially represent either ribavirin-induced mutations in occult chronically infecting populations or, less likely, potentially new independent HEV infections.

DISCUSSION

Chronic HEV infection in SOT recipients was first reported in 2008.[2] Since that time, infection in many profoundly immune suppressed cohorts have been documented.[1 3] HEV is likely under-recognised as many patients are asymptomatic or have non-specific abnormal liver enzyme tests. Assessment for chronic HEV infection in immunosuppressed populations requires the simultaneous assessment of both anti-HEV antibodies (IgG and IgM) and molecular assays to detect HEV RNA (owing to the potential for false negative serology in immunosuppressed hosts).[1 5] Unfortunately, chronic HEV infection can lead to rapid disease progression following acquisition.[1] Whereas the majority of cases have been reported in Europe, several North American cases have more recently been described.[6 7] Among chronic HEV infections in SOT, few have been reported in lung transplant recipients, including three chronic cases of HEV infection (3.2% of the total cohort reported)[8] and one case of HEV-associated meningoencephalitis in antecedent idiopathic pulmonary fibrosis.[9] To this point, other groups have not investigated an HEV chronic infection predilection in other cohorts such as pwCF. However, as 20% of adults with CF are lung transplant recipients, and lung transplant recipients are among the most heavily immunosuppressed SOT populations, understanding factors associated with adverse outcomes, such as HEV infection, are of the utmost importance.

HEV infection in high-income countries is predominately believed to be acquired through direct contact with pigs or the ingestion of undercooked pork, but as the vast majority of infections are asymptomatic, clinical diagnoses are rare.[10] This is well illustrated by the very low frequency of clinical testing reported here among the 4.5 million individuals living in Alberta, Canada's fourth most populous province. In contrast, a systematic review of HEV infection in the Americas observed a pooled seroprevalence ranging between 3.4% and 10.7% suggesting exposures over a lifetime do occur.[11] An important study of nearly 14 000 Canadian blood donors were tested for HEV RNA.[12] None of the specimens were HEV RNA positive. A subset was

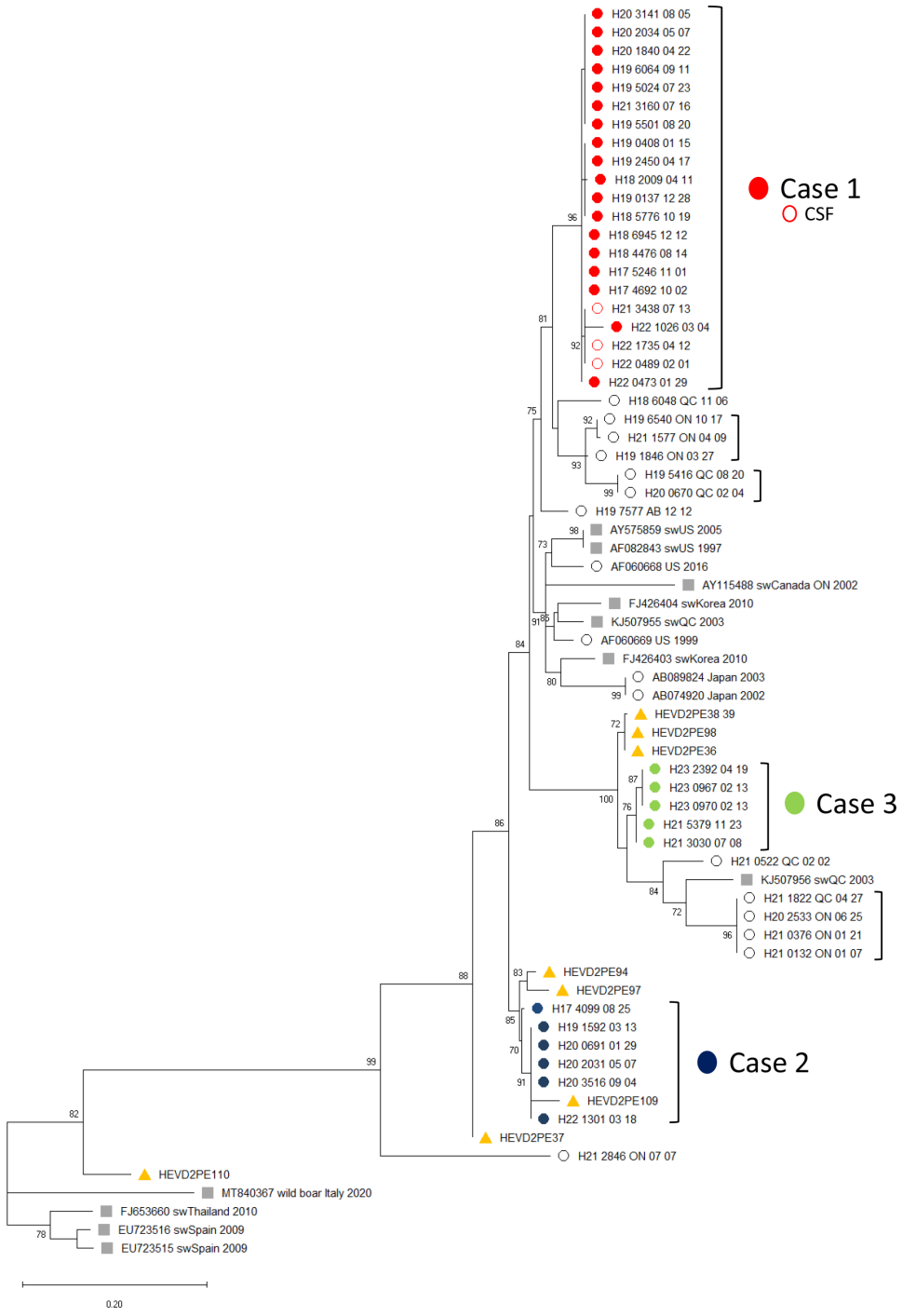

Figure 4 Phylogenetic analysis of hepatitis E virus (HEV) sequences from lung transplant patients. HEV sequence from consecutive, longitudinal specimens from the three cystic fibrosis (CF) lung transplant recipients (filled circles derived from plasma, open circles from cerebrospinal fluid (CSF)) were aligned with reference sequences including porcine-derived HEV genotype 3 (grey squares) and non-CF human HEV genotype 3 sequences (open circle). A total of 68 *orf1* sequences (8 pancreatic enzyme replacement therapy, 32 serial samples from 3 persons with CF lung transplant patients, 10 swine sequences, 1 wild boar sequence and 17 human sequences (16 acute travel-associated HEV and 1 previously not reported chronic HEV, including 13 from Canada)), were aligned, trimmed to 315 bp and analysed by maximum likelihood phylogenetic inference. Brackets denote sequences from the same individual. Those brackets without case numbers next to the cluster indicate non-CF human samples. Porcine and human HEV reference sequences show the GenBank accession number and the location and year of collection. Human sequences originating in Canada show the year ('H' number), followed by the patient 4-digit ID code, followed by the province and month and day of collection. The ruler shows the branch length for a pairwise distance equal to 0.2. Branch support by the approximate likelihood ratio test >70% is shown at branch nodes.

tested for HEV IgG and positivity rates ranged from 1.8% in Nova Scotia/New Brunswick to 6.7% in Quebec (average 5.9%). Seropositivity rates were highest in men, those working with farm animals and those born outside of Canada. In contrast, we observed uncharacteristically high rates of HEV seropositivity in pwCF, irrespective of transplant status, twice that even of those suspected of HEV (ie, those Albertan's screened for HEV) and fourfold higher than the general Canadian population. Notably

in our cohort, there was no association between rural postal code and HEV seropositivity, and all three individuals with chronic HEV infection were Canadian-born urban professionals and none reported ever eating raw or uncommon (ie, liver) pork products suggesting alternate risk factors.

Whereas blood transfusions have previously been evaluated as a risk factor for HEV transfusion-transmission in SOT recipients, we do not believe they were a contributing factor in pwCF. This is relevant as those undergoing lung transplantation almost universally receive blood products at the time of surgery.[13] A recent large study in the USA and Canada of over 100 000 donations found the frequency of HEV RNA was exceedingly low in blood products, with ~1:17 000 in the USA and ~1:4600 in Canada (p=0.062).[14] A risk-based decision-making process activity was undertaken by Canadian Blood Services to assess risks for HEV infection with different types of transplants. The estimated rate in heart and lung transplant recipients (lung transplant were not parsed out from heart transplants) of products in Canada (except for Quebec) was once every 711 years.[15] As CF accounts for a mere 2% of SOT surgeries each year in Alberta (~20% of lung transplant surgeries are for CF, and lung transplants comprise only 11% of SOTs (n=53/495)),[16] and all our observed cases of chronic HEV infection were observed in CF lung transplant recipients, this represents a significant outlier. Importantly, in the CF transplant cohort, the number of blood products received by HEV seropositive pwCF, including those with chronic HEV infection did not differ from seronegative and none received products after the transplant procedure itself. Finally, the timeline of transfusion as a risk for HEV infection is also inconsistent, as pwCF eventually diagnosed with chronic HEV infection first developed liver enzyme abnormalities many years after transplantation (the time of last transfusion) whereas median time to liver enzyme abnormality after incident infection has been estimated to be a mere 2–6 weeks in other cohorts.[10]

We postulated the only particularly unique feature of pwCF relative to other populations that may predispose to HEV, a zoonotic infection mostly commonly acquired from pigs, is the high frequency of PI (~90%). In fact, CF was first recognised and ultimately named on the basis of the pathological appearance of the pancreas at autopsy.[17] Sixty per cent of pwCF are born with PI and up to 90% will become PI by 1 year of age.[18] PI is managed through exogenous porcine-derived PERT administered in capsules where lipase, protease and amylase enzymes are packaged into granules or microspheres coated with a pH-sensitive matrix preventing enzymatic degradation in the stomach and enabling their release in the alkaline environment of the duodenum.[19] Whereas PERT was initially exempt from Food and Drug Administration (FDA) approval in 1938, in 2006, new requirements for manufacturing and safety were introduced with all currently approved formulations derived from pigs.[20] Adverse events attributed to PERT have rarely been described, with the exception of the potential for fibrosing colonopathy in young pwCF receiving >10 000 U lipase/kg/day.[21] In fact, UK consensus guidelines for management of PI-indicated PERT is not associated with any significant complications (grade 1A, 100% agreement).[22] Moreover, the FDA's PERT guidance statement outlines 'it is not necessary to conduct long-term safety evaluations of PERT in support of new drug applications; this is because of the long and extensive safety experience with PERT'. However, the potential for zoonotic transmission of viruses through porcine-derived PERT was always recognised.[23]

Potential safety mechanisms in PERT production presumed to mitigate zoonoses risk relate to certificates of animal health, acceptance criteria and viral load testing, viral inactivation studies and surveillance for animal diseases.[24] However, a study of Canadian commercial swine herds (~1000 animals) demonstrated that by 6 months of age (ie, time of slaughter) 60% were HEV seropositive, with HEV RNA detected in the faeces of many.[25] Furthermore, pigs infected with HEV are asymptomatic[26] and can shed virus for ≥60 days.[27] In porcine models of HEV infection, the pancreas, in particular the acinar cell-rich areas (from which PERT is derived), are disproportionally infected.[28] HEV is highly resilient, as demonstrated by its ability to be transmitted in a range of pork products that are not properly cooked at >71°C for >20 min.[29]

While studies involving recipients of HEV-infected blood products suggests the lowest HEV infectious doses ranges from 7000 to 36 000 IU depending on the product,[30] the required infectious dose in profoundly immunosuppressed hosts is likely to be markedly lower.

In our study of independently sourced PERT, we found 44% to be HEV RNA positive. For the average pwCF consuming 24 PERT capsules per day (median of the cohort), this would equate to 3680 HEV RNA-positive PERT capsules consumed per year. If even 1/10 000 of these RNA-positive PERT capsules had infectious HEV, it would still equate to ~0.4 exposures per person per year in our cohort. Furthermore, as multiple PERT are consumed at a time the risk posed by any individual capsule is enhanced through cumulative dosing. While our extraction protocols were designed to mitigate the extremely protein dense matrix of PERT and compensate for its enteric coating, high levels of PCR inhibition were identified suggesting that rates of PERT positivity and amount of HEV RNA identified here are likely underestimated. Indeed, we were able to sequence both HEV *orf1* (multiple segments) and *orf2* loci despite the relatively low levels of HEV RNA detected by RT-qPCR. We were not surprised to have been unable to confirm HEV by either whole genome sequencing, capsid protein assessment or in vitro cell culture assay given the established insensitivities of these modalities relative to their molecular counterparts and the challenging nature of the PERT matrix.[31] Furthermore, in vitro testing of PERT is likely to be vastly underpowered to detect viable HEV given the fact that the assessed 107 PERT capsules are the equivalent to a mere ~4.5 days of use by the average pwCF—and we would expect the frequency of viable HEV contaminating PERT to be very rare given the few clinical cases identified. Owing to the intrinsic challenges of performing molecular and genomic testing on the protein dense and inhibitor-rich PERT capsules, we believe that the best means to definitively prove the potential of HEV acquisition from PERT would be through controlled animal challenge experiments—allowing for the longitudinal and cumulative exposure of individual subjects to the large quantities of PERT required to identify these exceedingly rare acquisition events.

Despite our hypothesis that PERT represents an HEV exposure risk disproportionate to pwCF, and our confirmation that HEV RNA does contaminate a high proportion of PERT, the risk posed by any individual PERT capsule must still be remarkably low explaining the small numbers of cases observed here. However, this does not diminish the potential scope of HEV contamination of PERT. It is estimated there are ~105 000 diagnosed individuals living with CF worldwide,[32] 90% of whom use PERT. Among these, 519 and 1645 individuals are transplant recipients (~97% lung) living in Canada[4] and the USA,[33] alone. Furthermore, there are many other conditions for which PERT are routinely prescribed (ie, postpancreatectomy and those with chronic pancreatitis). The scope of zoonotic risks from PERT may not be confined merely to HEV. Other porcine-derived

human zoonoses may also be possible including established agents such as Japanese encephalitis virus, Nipah virus and swine/avian influenza strains, and others that could potentially infect humans including porcine coronaviruses, circovirus and parvovirus.[34] Seroprevalence studies assessing for disproportionate exposure risk of these agents could be considered in CF and other PERT-using populations.

A potential explanation as to the absence of other reported cases of HEV infection in CF may relate to the very high frequency with which diseases of the liver exists in CF, with CFLD present in up to one-third of individuals.[35] Whereas classic CFLD is attributed to ductal cholestasis, resulting in inflammation and periportal fibrosis, several other presentations are observed including hepatic steatosis and focal biliary and multilobar cirrhosis. Furthermore, abnormal liver enzymes are observed frequently through longitudinal observation, including in >90% of children with CF.[36] Accordingly, clinicians may simply fail to investigate alternate causes of liver enzyme rise, and progressive liver disease, such as HEV.

The management of chronic HEV infection in SOT recipients is complex, and clinical practice guidelines have been developed to guide therapy.[37] Some chronically infected individuals will clear infections spontaneously. The first step proposed by many experts is reducing immunosuppression, a strategy that has been associated with treatment success in other SOT recipients.[38] Dose reduction in maintenance immunosuppression was not undertaken here owing to concerns held by lung transplant care providers about potential allograft rejection. In many cohorts, treatment with ribavirin is effective and sustained virological response rates as high as 78% have been reported within 3 months in some studies,[39] despite none having achieved here. Individuals in our cohort received consistent ribavirin treatment, and dose reductions were avoided as late as possible unless haemoglobin levels fell below 90 g/L with associated prostrating fatigue. Significant nausea and vomiting were universally observed and proved challenging for the patients. While two individuals demonstrated treatment-related viral clearance (having received treatments much longer than conventional published cohorts), both relapsed/recurred or were reinfected. Moreover, the first case did not ever demonstrate any significant reduction in viral loads despite normalisation of liver function tests. Importantly, no mutations associated with ribavirin resistance, including G1634R, were identified in any patient samples.[40] While it is unknown if specific HEV genotypes may show differing treatment responsiveness to ribavirin, the HEV 3a strains observed here are uncommonly identified in European populations where treatment success rates appear to be higher.[41]

We recognise several limitations of this work. First, as a cross-sectional, single-centre study, we were unable to determine timing of acquisition and spontaneous clearance of viraemia in those HEV with seropositivity, but negative for HEV RNA. Because of the nebulous time frame, we did not perform a formal risk exposure questionnaire across cohorts which could help identify past exposures to potential sources of HEV in future surveillance studies. While other studies have previously associated HEV seropositivity risk with ingestion of pork products (ie, bacon and cured meats),[42] it is important to note that HEV RNA has not been identified in commonly consumed commercial meat products such as rib, bacon, lean ham and loin.[43–46] In stark contrast, we observed HEV RNA in 44% of all PERT capsules screened (equating to 10 HEV RNA-positive capsules consumed by each pwCF daily). We acknowledge the small sample size of our CF cohort, but we sought to mitigate this by including all CF transplanted individuals and complimentary control groups including a larger non-transplanted CF population residing in the same area and >1000 non-CF controls (ie, every patient in our province, with a population of >4.5 million individuals, where HEV infection was queried over the last 7 years). Importantly, aside from our transplanted pwCF cases, no other cases of chronic HEV infection were identified in any other cohort we evaluated. As this was a single-centre study, this may not be representative of other CF and transplant centres as there may be inherent geographical differences in prevalence of HEV.[11] Most importantly, while we have identified HEV RNA in 44% of all PERT capsules screened (including samples from all four Canadian manufacturers), we have not confirmed replication-competent HEV.

CONCLUSION

Here, we describe the first cases series of chronic HEV infection identified in Alberta (and Canada)—all in pwCF post-transplant. Only one other HEV chronic infection case has previously been published in a Canadian liver transplant recipient,[47] and none other have been identified in Canada's single National reference laboratory. Treatment proved exceedingly challenging with no case cured, one death and significant morbidity experienced by all. The identification of disproportionate HEV seropositivity among pwCF and a high prevalence of HEV RNA contaminating PERT (a commonly prescribed FDA-approved, European Medicines Agency-approved and Health Canada-approved therapeutic class) suggests a potential iatrogenic mechanism of HEV acquisition that must be further explored. Screening for HEV infections in pwCF post-transplant and establishing the seroprevalence of HEV in other cohorts of pwCF are urgently required.

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Contributors CST, BJW, MDP planned the study. BJW, JS carried out laboratory analysis. CST, BJW, SEC, JS, LF, RS, KF, DI, KD, SJD JB, CO and MDP gathered and analysed data. CST, BJW and MDP wrote the first draft of the manuscript. All authors contributed to revisions of the final draft. MDP is the guarantor of the work.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study was approved by the University of Calgary's Conjoint Health Research Ethics Board (REB22-1216). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

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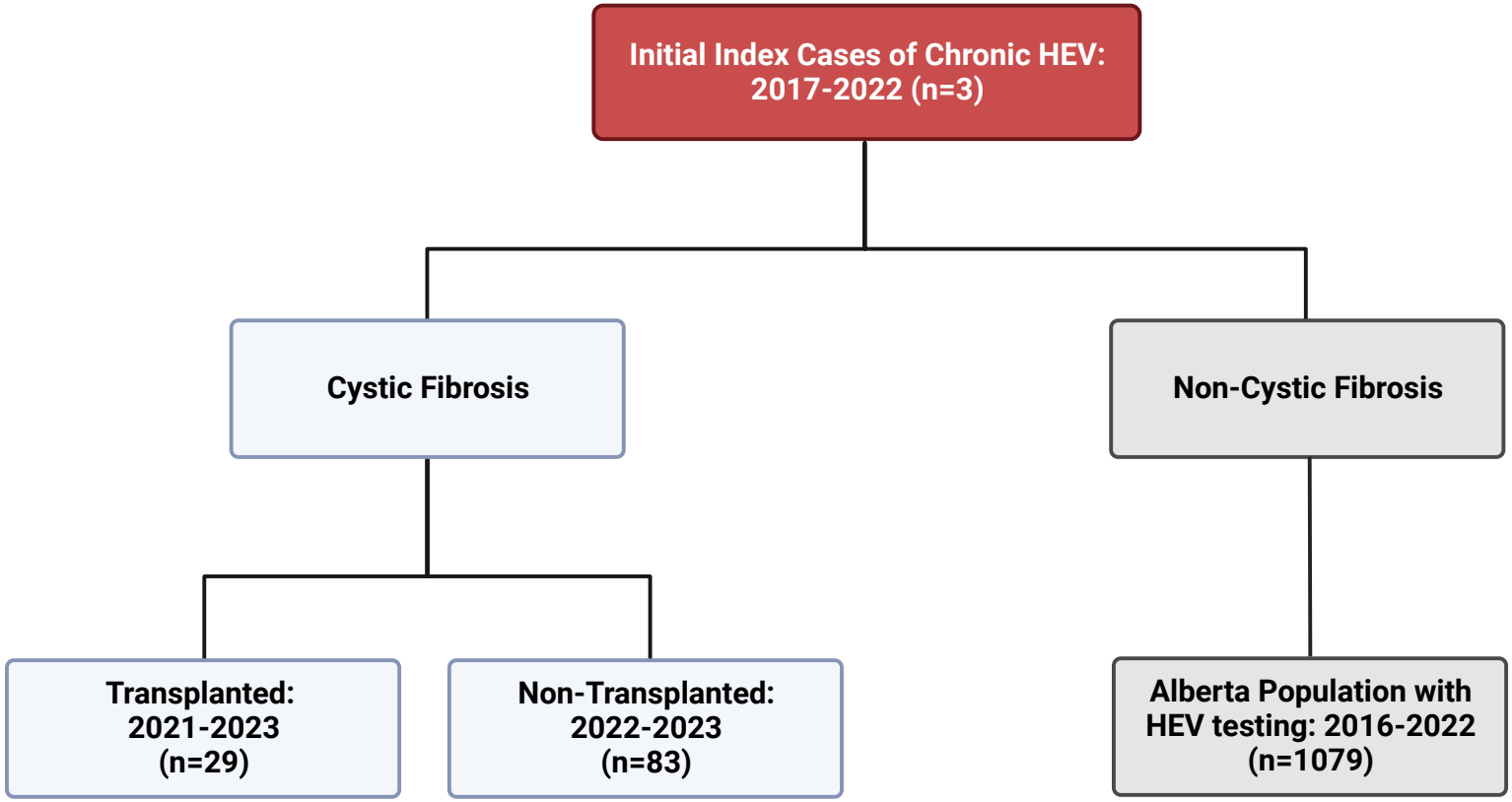

ONLINE SUPPLEMENT

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^{TM} 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^{TM} was mixed with dissolved aliquots as per the TRIzol^{TM} reagent instruction for RNA purification by vortexing for

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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

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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.

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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

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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 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% 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 culture. Cells were split on day 15 to check by immunofluorescence.

SUPPLEMENTARY RESULTS

HEV Serological Testing - ELISA Details

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

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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

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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.

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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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