Hepatitis E Virus Seroprevalence in Canada

by

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Abstract

Hepatitis E virus (HEV) is the main cause of acute viral hepatitis worldwide, predominantly in developing areas where it is endemic. The disease is generally self-limiting with many individuals being asymptomatic, however, immunocompromised individuals or patients with pre-existing liver disease may experience much higher rates of morbidity and mortality. Recently, HEV has gained more attention in the developed world. This has prompted several industrialized countries to assess seroprevalence rates using blood donor samples. HEV has the potential to be a serious threat to the blood safety, and careful monitoring is essential to maintain blood safety and prevent transfusion-associated transmission of the virus to the most susceptible individuals. Currently, no such data exists for Canada, and the prevalence of this virus in the general population is largely unknown.

The focus of this study is to (1) determine the seroprevalence of HEV in the Canadian blood donor population. (2) Apart from the transfusion associated risk, we will also assess the prevalence of HEV among patients considered to be at a higher risk for acquiring the infection: individuals with compromised immune systems due to treatment and or illness, and people who inject drugs. (3) We will also evaluate the potential role of wild animals, such as different deer species, as a reservoir for HEV as this could represent another important source of infection for humans.

The overall seroprevalence among Canadian blood donor samples collected from July 2013 - December 2015 was 5.84% (240/4,107), and none of the 14,053 samples tested in pools of 48 or 96 were positive for HEV RNA. There was no significant increase in the high-risk groups we tested other than the patients receiving plasmapheresis, but this could be due to passive transfer of antibody. HIV was

determined to be a significant risk-factor for HEV infection in a retrospective study of Kenya-based sex-worker cohorts, but not so in a Canadian cohort of HIV-positive intravenous drug users. HEV infection occurs in deer, however, further investigation is needed to evaluate the zoonotic risk to humans. Overall HEV seroprevalence (in blood donors) in Canada is lower than that published in other countries; this together with the fact that we failed to detect HEV RNA in Canadian blood donations indicates that HEV currently poses a low risk to the Canadian blood supply.

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List of Abbreviations

ALT- Alanine aminotransferase

ALP - serum alkaline phosphatase

AST- Aspartate aminotransferase

CBMTG- Canadian Bone Marrow Transplantation Group

CBS- Canadian Blood Service

cDNA- Complementary deoxyribonucleic acid

CWHC- Canadian Wildlife Health Cooperative

DNA- Deoxyribonucleic acid

ELISA- Enzyme-linked immunosorbent assay

GGT- gammaglutamyl amino transferase

HAV-Hepatitis A Virus

HBV- Hepatitis B Virus

HCV- Hepatitis C Virus

HEV- Hepatitis E Virus

HIV- Human Immunodeficiency Virus

HRP- Horseradish peroxidase

IC- Internal control

IDU- Intravenous drug user

IFN- Interferon

IgG- Immunoglobulin G

IgM- Immunoglobulin M

mmol- Millimole

NAT- Nucleic acid testing

NK- Natural Killer

ORF- Open reading frame

PCR- Polymerase chain reaction

PEG- Pegylated

RdRp- RNA-dependent RNA Polymerase

RNA- Ribonucleic acid

RT-PCR- Reverse-transcriptase polymerase chain reaction

HQ- Héma-Québec

TTP- Thrombocytic Thrombocytopenic Purpura

UTR- Untranslated Region

1. Introduction – Hepatitis E Virus Literature Review

Hepatitis E Virus:

Hepatitis E Virus (HEV) belongs to the family Hepeviridae, which is divided into two genera: Orthohepevirus (all mammalian and avian hepatitis E virus isolates) and Piscihepevirus (cutthroat trout virus). Species within the genus Orthohepevirus are designated Orthohepevirus A (isolates from human, pig, wild boar, deer, mongoose, rabbit and camel), Orthohepevirus B (isolates from chicken), Orthohepevirus C (isolates from rat, greater bandicoot, Asian musk shrew, ferret and mink) and Orthohepevirus D (isolates from bat)[1–3].

HEV is the main cause of acute viral hepatitis worldwide, causing an estimated 20 million cases each year resulting in 70,000 deaths and 3,000 stillbirths [4]. It causes symptoms typical of acute-viral hepatitis including jaundice, malaise, fever, abdominal pain, and liver inflammation. The virus commonly infects young healthy subjects, however, the disease is generally self-limiting with many being asymptomatic [5]. The mortality rate ranges from 0.2 - 4.0% [4]. HEV is associated with a very high morbidity and mortality in pregnant women and the fetus, particularly in the third trimester. In these clinical cases, acute liver failure and death may occur in up to 20-25% of these women, particularly in developing countries[6,7]. The underlying factors influencing this high mortality during pregnancy is poorly understood [8]. Patients with underlying chronic liver conditions such as cirrhosis, as well as immunocompromised individuals also experience higher rates of mortality [9]. The infection can also become chronic in immunocompromised patients, and eventually cause liver cirrhosis and eventually liver failure [9].

1.1 Hepatitis E Virus History:

The first known outbreak of HEV occurred in India around 1955-56, and affected as many as 29,000 individuals [10]. However, it was not until much later that the identity of the causative pathogen was determined. In 1978, Hepatitis E Virus was recognized during an epidemic of Hepatitis occurring in the Kashmir Valley of India[11], affecting an estimated 52,000 people resulting in 1700 deaths. There were several unique characteristics associated with this outbreak that led to the belief that there may be another non-A, non-B hepatitis virus. First, the epidemic was water-borne with a highly compressed epidemic curve suggesting a single source of spread it; and following the epidemic, secondary waves of hepatitis did not occur[12]. Young adults made up the majority of the cases, and there was a noted increased severity of the disease in pregnant women[12]. All of the surviving patients had self-limiting disease that resolved without intervention, and their sera lacked the serological markers for acute hepatitis A and hepatitis B[13].

Balayan and his research team definitively described the discovery of the new non-A, non-B hepatitis virus in the early 1980's during the Soviet occupation of Afghanistan. Balayan ingested pooled fecal extracts from 9 affected soldiers, and subsequently became ill. The new virus, named HEV, was detected in his stool using electron microscopy[14]. In 1990, Reyes et al. successfully cloned and sequenced the hepatitis E virus genome [15]. In the years that followed, research into this newly discovered hepatitis virus continued and it was identified as a major health concern in developing countries with poor sanitary practices and unsafe water supplies[11]. Recently, more attention has been focused on the zoonotic transmission, and its

increased prevalence in developed countries. This is due to ingestion of raw or undercooked meat of the infected animals, and animal products such as pig livers or sausages made from the livers and sold in supermarkets[11].

1.2 Hepatitis E Virus Molecular Biology:

Hepatitis E virus is a small non-enveloped virus, about 27-34 nm in size, present in the bile and feces of infected hosts [15]. The genome is 7.2 kb in length; consisting of positive-sense, single-stranded RNA, 7-methylguanine capped at the 5' termini and polyadenylated at its 3' termini[11]. There are three open reading frames (ORF) encoded by the HEV genome, as well as 5' and 3' untranslated regions (UTR) of approximately 58 and 68 nucleotides respectively [8]. These UTR's have been shown to fold into stem-loop structures [16]. Experiments have shown these stem-loop structures are required for replication, and abolishment of these sequences prevents binding of the RNA-dependent RNA polymerase (RdRp) and subsequent transcription[17]. ORF1 encodes a non-structural polyprotein of 1693 amino acids in length, containing domains and functional motifs such as protease, methyltransferase, RdRp, and RNA helicase [15]. ORF2 encodes the major viral capsid protein, 660 amino acids in length. This protein is involved in virion assembly, the subsequent association with target cells, and also induces neutralizing antibodies blocking HEV infection [1]. The ORF3 protein is very small, 113-114 amino acids in length, and until recently its function was unknown. Research published in 2008 by Chandra et al. suggest that it acts as an adaptor to link the intracellular transduction pathways, reducing the host inflammatory response thereby acting to protect the cells infected with HEV virus [18]. This protein has also been shown recently to be important for viral particle egress from the infected cells [16,19,20].

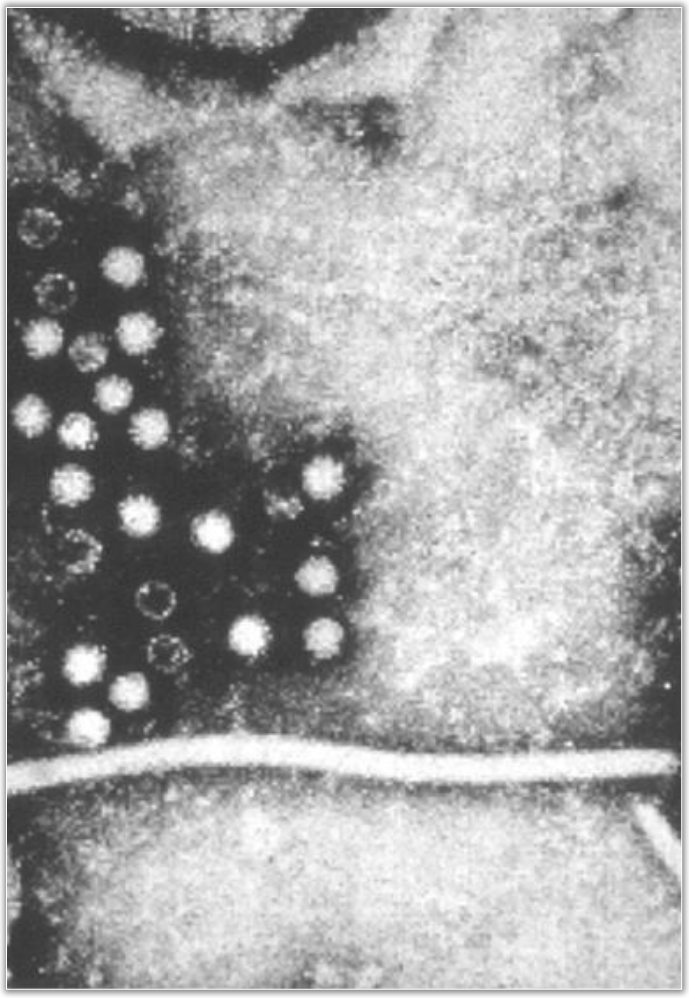

Figure 1: Electron Microscopy of Hepatitis E Virus particles (CDC Public Health Image Library)

HEV particles found in circulating blood and culture supernatant are covered in a type of lipid membrane similar to enveloped viruses and are poorly neutralized by anti-HEV positive immune sera or anti-ORF2 monoclonal antibody [20–22]. Experiments comparing HEV particles taken from serum and culture supernatant, bile duct (containing deoxycholic acid), and in feces to have sucrose gradient density of 1.15g/ml, 1.21-1.24g/ml, and 1.24g/ml respectively [23]. The increased density is owing to the fact

the particles are naked and not semi-enveloped in lipid membrane. The mechanism by which the virus becomes covered in lipid-like semi-envelope is unknown. A diagrammatic representation of the exterior virus particle in various physiological locations within the body is shown in Figure 2.

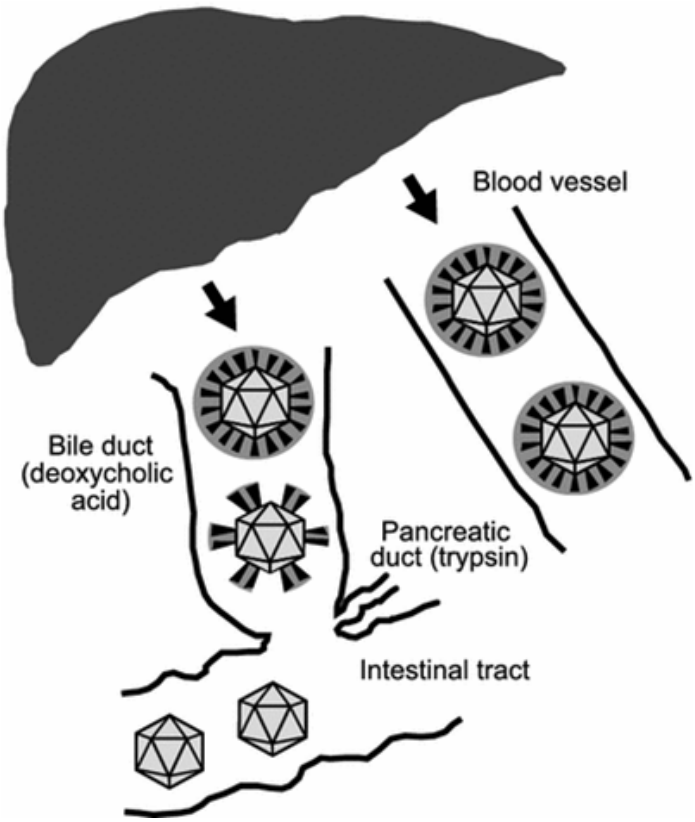

Figure 2: Hepatitis E virus particle representation in various locations within the body [23].

Due to the lack of a suitable cell culture system for HEV, the life cycle and replication steps therein have not been easily elucidated. The proposed process for HEV replication commences with the virus attaching to the surface of hepatoctyes. The virus

binds to an unknown receptor, uncoats, and finally releases its genomic RNA into the hepatocyte, where translation will occur in the cytoplasm. RdRp synthesizes negative-sense RNA from the positive-sense genome. This will subsequently be used as the template for synthesizing subgenomic RNA and full-length positive-sense transcripts. ORF2 and ORF3 proteins are translated from the subgenomic RNA transcripts, and are responsible for virus assembly as well as conditioning the host environment for HEV replication [1,24]. The mechanism of HEV egress from the host cells remains unknown[25].

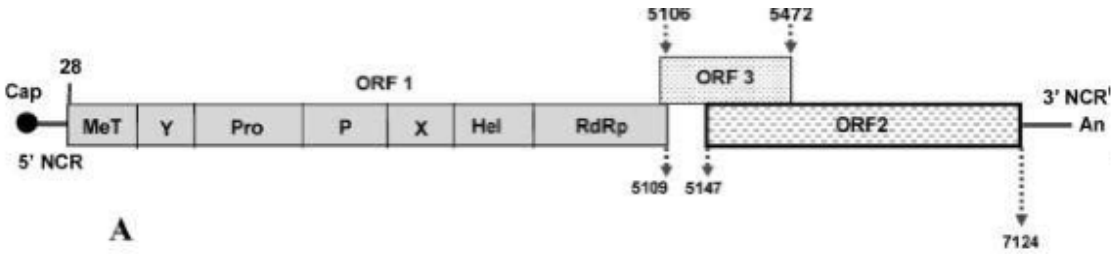

Figure 3: Genomic Organization of mammalian HEV[26].

1.3 Immune Response to Hepatitis E Virus

The immune response that is initiated against HEV upon infection involves the recruitment of immune cells to sites of viral replication. Initially, these immune cells utilize pattern recognition receptors in the early stages of the infection. Type 1 interferons (IFN's) produced within the cell are pro-inflammatory cytokines that are essential to the antiviral response following the initial recognition of viral infection [27]. During HEV infection, interferon-inducible genes are upregulated. This is important because Type 1 IFN's serve important roles in the innate immune response during viral infection.

Natural killer (NK) cells are involved in eliminating virus-infected cells. NK cells are activated by IFN-γ, which also activates some other T cell functions. HEV infected individuals have been shown to have increased CD8+ and CD4+ CD8+ T cells compared with healthy patients [8]. ORF2 and ORF3 proteins induce a higher proportion of IFN-γ secreting cells than in controls, proving the effector T cell response to HEV components [17].

Anti-HEV IgM antibody production occurs promptly following infection and levels increase rapidly up until about 3 months when they begin to subside[28]. Anti-HEV IgG on the other hand will persist for years after its production begins, shortly after the waning of anti-HEV IgM[29]. This rapid humoral immune response clears the virus and also provides immunity for the future. Laboratory diagnosis can be made using serological tests for anti-HEV IgM antibody. HEV RNA can be detected in patient blood and stool samples but only for a limited period of time[30]. Figure 4 illustrates the HEV infection-immune response. In a study using human volunteers it was found that HEV RNA could be detected in blood sooner than in feces; around 20 days after inoculation[31].

A complex network of innate and adaptive immune responses mediate protection against HEV. However, during a persistent infection, the immune response may also cause tissue injury while attempting to eradicate the virus. The pathogenic process of HEV infection is difficult to study, owing to the lack of a suitable cell culture system. Insight gained has relied heavily on recombinant HEV proteins rather than studying actual viral infection [8].

1.4 Clinical Manifestations of Hepatitis E infection, Laboratory Findings, and Diagnostics

The clinical manifestations of HEV infection range from subclinical to acute liver

failure and death. Mean incubation time is 40 days, but can range from between 15 to 60 days[31]. One study, using a human volunteer that had consumed infected fecal material, reported clinical symptoms after 36 days[14]. The infection may be completely asymptomatic, or may lack any distinguishing characteristics that may help identify it from any other viral febrile illness. Acute hepatitis caused by HEV is virtually indistinguishable from that of other forms of viral hepatitis. In patients who do experience symptoms from acute HEV infection, the clinical course runs a few weeks and is self-limiting in most healthy individuals. The average length of the pre-icteric phase (the phase preceding jaundice) is 3 to 4 days, but could last up to 10. During this time, gastrointestinal symptoms such as nausea, vomiting, and epigastric pain have been frequently reported. The appearance of jaundice heralds the beginning of the icteric phase, during which time dark urine and clay colored stools will also be typical. This phase lasts only 12 to15 days in most uncomplicated cases, with complete recovery taking place within 1 month as illustrated in Figure 4. During illness about two-thirds of patients complain of arthralgia (joint pain) and half of the patients develop a fever.

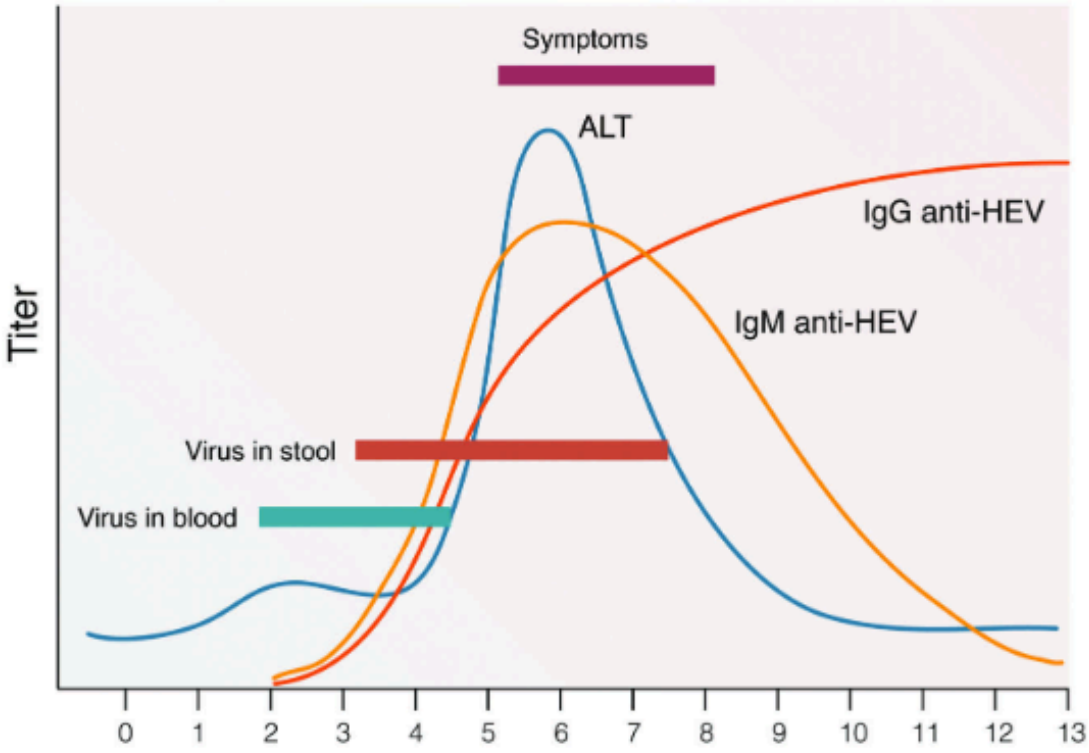

Figure 4: Hepatitis E virus infection-immune response and viremia weeks measured after infection[32].

In immunocompromised individuals and very rarely in some individuals who are healthy, acute hepatitis may run a prolonged and severe course. It may even cause hepatic failure, become chronic, or have extrahepatic manifestations; including pancreatitis, arthritis, aplastic anemia, and neurologic complications have all been reported[33–35 34–36]. Hepatitis E infection is particularly severe among pregnant women, elderly men, and also in individuals with a pre-existing chronic liver disease such as cirrhosis[66,37]. In addition, individuals with chronic liver disease may experience acute-on-chronic liver failure caused by acute HEV [38].

Laboratory test abnormalities include significant elevation in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Gammaglutamyl amino

transferase (GGT) and serum alkaline phosphatase (ALP) activities may increase but much less so. Hyperbilirubinemia and bilirubinuria will occur when severe hepatitis occurs. Liver function tests (bilirubin, albumin, and INR) will be helpful in determining if hepatic necrosis is present. As with most other forms of viral hepatitis, a single peak in ALT elevation is seen preceding or coinciding with the onset of jaundice[39]. HEV RNA is detectable in serum at about day 22 in humans and persists during the pre-icteric phase until it begins to decline and totally disappears by the peak of the ALT rise [40].

The diagnosis of acute hepatitis E infection can be made by having both clinically relevant symptoms of acute hepatitis combined with a positive anti-HEV IgM[41]. Acute HEV may also be diagnosed when one identifies a positive anti-HEV IgM antibody with no symptoms, refered to as subclinical hepatitis E. On the other hand, observing a persistent increase in liver enzyme levels as well as PCR-detectable HEV in the serum or stool over a 6 month period is indicative of chronic HEV infection [33]. Genotype 3 is particularly known to cause chronic infection among solid organ recipients and other immunocompromised individuals.

1.5 Treatment of Acute Hepatitis E Infection

Acute hepatitis E infection in immunocompetent patients usually only requires supportive care, as most individuals will clear the virus on their own. There is no specific treatment indicated, although ribavirin has shown significant clinical improvements in liver function enzyme levels and reduced the duration of symptoms [42]. Ribavirin carries inherent risks to the fetus, and is contraindicated during pregnancy due to its teratogenicity. However, the risks of untreated HEV to the mother and fetus are significant, so a course of antiviral treatment may be warranted [1]. Ribavirin therapy

may be also beneficial to individuals with underlying chronic liver disease, as this population has a high risk of fulminant liver failure and mortality caused by an acute HEV infection in acute-on-chronic liver disease. Early treatment is also necessary for immunosuppressed individuals and those receiving solid organ transplants due to the high likelihood of the infection proceeding into chronicity [42–44]. There have been a couple of studies investigating the proportion of solid organ transplant recipients who went on to develop chronic HEV infection, and was found to be from 58-66% compared to the control group[33,45]. Immunosuppressive drugs such as tacrolimus are the main risk factor for the progression to chronicity, and reducing immune suppression will enable about 30% of these individuals to clear the virus[46,47] .

Sometimes reducing the immunosuppression isn't possible, or the patient is still unable to clear the virus after reducing immunosuppressive therapy. In these cases, there are two additional treatment options that may be studied. The first option is using Ribavirin monotherapy at a dose in the range of 29-1200 mg/d determined by the patient's creatine clearance and body weight (median dose 600mg) for at least 3 months, up to as long as 18 months[44,46,48]. One study reported that 95% of patients experienced HEV clearance at the end of the ribavirin therapy, and 78% had a sustained virological response with no serum HEV- RNA detectable 6 months after the therapy ended [48]. The median duration of ribavirin monotherapy in this study was 3 months. It has been suggested that ribavirin exerts its antiviral effect against HEV by depleting intracellular guanosine 5'-triphosphate pools, but this mechanism is still poorly understood and requires further research to elucidate this function [49]. The data has shown that although ribavirin may contribute to hemolytic anemia in some individuals, it does provide therapeutic benefit to patients suffering from chronic hepatitis E.

Because ribavirin treatment requires close monitoring of hemoglobin levels, it makes it hard to implement this therapeutic regimen in developing countries with scarce resources and inadequate healthcare systems[50].

The second option is to use pegylated (Peg)-IFN- α for between 3 to 12 months[47,51]. Reports have shown that desirable liver function enzyme levels have been achieved as well as viral RNA suppression with this treatment for solid organ transplant recipients. However, some of these individuals developed allograft rejection after Peg-IFN treatment, particularly in those who have received heart or kidney transplantation [52]. Some patients also experience severe flu-like symptoms after Peg-IFN treatment [8]. Successful combination therapy has been reported in one HIV-positive patient with chronic HEV infection, and synergistic effects of ribavirin with Peg-IFN- α was demonstrated in one in vitro study[49,53]. This may allow clinicians to lower the ribavirin dosage in patients experiencing anemia and other treatment related side-effects[49].

Another treatment currently being investigated for use in immunocompromised patients with chronic HEV infection is sofosbuvir, an antiviral drug used for HCV. Sofosbuvir is an oral prodrug of a nucleotide hepatitis C virus RNA-dependent RNA polymerase inhibitor [54]. A recent project has shown sofosbuvir to efficiently inhibit HEV-RNA replication in HEV3 Rep/Neo cells (IC50, 1.2 mmol/L), using HEV replicons generated from HEV genotype 3, and has an additive effect when combined with ribavirin [55]. This drug may prove to be a more effective treatment option (or add-on therapy) for chronically infected individuals, and in those immunosuppressed individuals unable to clear the virus with ribavirin alone.

1.6 Hepatitis E Prevention

There is currently no FDA approved vaccine for use against HEV in North America; however, one was recently released for use in China. HEV 239, or "Hecolin," recently developed by Xiamen Innovax Biotech is an HEV vaccine based on bacterial recombinant ORF2 antigen using genotype 1. A large randomized, double-blind, placebo-controlled phase 3 trial has proven it to be both safe and effective in healthy adults in China's general population [56,57]. The study used healthy individuals ranging in age from 16-65 years old. Close to 100,000 participants were randomly allocated to receive 3 doses of either Hecolin or placebo (hepatitis B vaccine) intramuscularly at 0, 1 and 6 months. The endpoint assessed was prevention of HEV infection at 12 months from day 31 of the third dose. 15 individuals in the placebo group developed HEV infection compared to none in the vaccine group, demonstrating an efficacy rate of 100% after 3 doses of vaccine. There were no serious adverse reactions to the vaccine reported, and it was well tolerated by the participants.

The major limitation of the study was the exclusion of individuals with chronic liver disease, as this group should be prioritized to receive HEV vaccine. Due to the lack of an HEV case in the vaccine, another limitation was that protective antibody concentration could not be assessed. Further studies aimed at assessing the safety and efficacy of Hecolin in pregnant woman, as well as children aged 15 years and younger, and adults older than 65 years are needed. Although this study proves that this vaccine may be helpful in highly endemic regions to reduce sporadic and epidemic spread of HEV, for developed countries it is of little interest as HEV infection, although on the rise, is still not a major problem. There is thus no urgency to fund or include it in immunization regimes here in Canada or the United States.

1.7 Hepatitis E Virus Classifications and Epidemiology:

HEV was first classified within the Calicivirdae family of viruses due to its similarity to the Norwalk virus in morphology and biophysical properties [58]. It was then transferred into the Togaviridae family after phylogenetic analysis of the RNA-dependent RNA-polymerase, helicase and other positive stranded RNA viruses found it more related to Rubeola virus [58]. Today, it is classified within the Hepeviridae family as a single serotype[28]. Identification of novel strains in various animal species causes the HEV nomenclature to be debated and changed frequently. Table 1 illustrates the most recent classification scheme which divides the Hepeviridae family into two genera; Orthohepevirus and Piscehepevirus[59]. The variants of Hepatitis E virus that infect humans are classified into 4 of the 7 genotypes (HEV1-4) contained within the Orthohepevirus A species. Each has several subtypes, based on analysis of viral genomic sequences[60–62 60–62]. Genotypes 1 and 2 (HEV-1 and HEV-2) only infect humans, while genotypes 3 and 4 (HEV-3 and HEV-4) have been detected in humans as well a number of other animal species[63]. Between genotypes 1 to 4, genetic similarity ranges from 73 to 77%, while within each genotype it is over 81%[64]. There is much greater nucleotide sequence homology within genotype~~s~~ 1, while genotypes 3 and 4 are much more diverse. Figure 5 shows a schematic representation of neighbor-joining phylogeny for different HEV genotypes and subgenotypes.

Table 1: Proposed classification of the family Hepeviridae [65].

Proposed classification of the family *Hepeviridae*

Family	Genus	Species	Prototype isolate	GenBank accession	Predominant host species	Genotype	Reference strain	Reference accession
Hepeviridae			Burma	M73218	Human			
	Orthohepevirus		Burma	M73218	Human			
		Orthohepevirus A	Burma	M73218	Human	HEV-1	Mexico	M73218
					Human	HEV-2	Burma	M74506
					Human, pig, rabbit, deer, mongoose	HEV-3	Meng	AF082843
					Human, pig	HEV-4	T1	AJ272108
					Wild boar	HEV-5	JBOAR135-Shiz09	KJ496143
					Wild boar	HEV-6	wbJOY_06	AB573435
					Camel	HEV-7	DcHEV-178C	AB602441
		Orthohepevirus B	F93-5077	AY535004	Chicken			
		Orthohepevirus C	R63	GU345042	Rat	HEV-C1	R63	GU345042
					Ferret	HEV-C2	FRHEV4	JN998606
		Orthohepevirus D	BatHEV/BS7/GE/2009	JQ001749	Bat			
	Piscihepevirus		Heenan Lake	HQ731075	Trout			
		Piscihepevirus A	Heenan Lake	HQ731075	Trout			

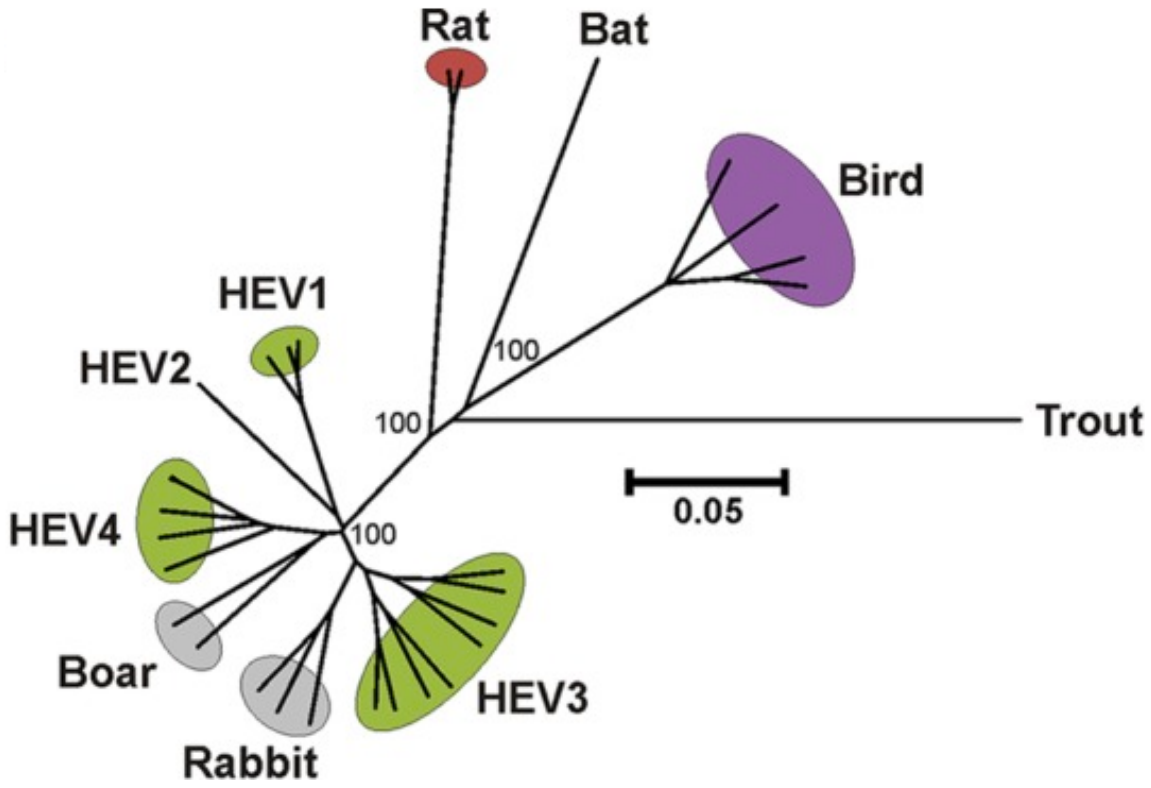

Figure 5: Neighbour-joining phylogeny of the complete genomes of members of the Hepeviridae using the nucleotide percentage distance substitution matrix and complete deletion option in MEGA5. Values at deep node points indicate support from 1,000 bootstrap reiterations; those at apical nodes are hidden for clarity of presentation [66].

HEV is a major cause of hepatitis outbreaks transmitted through the fecal-oral route [67]. Often in these developing areas, people use a common source for cooking, drinking, and bathing. HEV RNA has been detected in outbreaks in the sewage-contaminated water source [8]. In places lacking adequate public infrastructure, such as in refugee or military camps, this becomes a serious limitation that facilitates the spread of HEV infection [68]. Natural disasters including earthquakes and monsoon storms can also facilitate HEV epidemic outbreaks. Displaced populations in these situations, having only limited access to clean water and lacking sanitation facilities, who are naïve

immunologically to HEV, will have much higher rates and risks of transmission [69].

Genotypes 1 and 2 are transmitted via the fecal-oral route, and only infect humans. It's estimated that approximately 21 million people are infected with genotypes 1 or 2 annually [8]. These genotypes are restricted to developing areas with poor sanitation practices and risky water supplies where HEV is most common. Boiling and chlorination of water will inactivate the virus. These genotypes cause the epidemic outbreaks of acute hepatitis E [68]. Of the HEV isolates obtained from industrialized countries that are of the Genotype 1 or 2 varieties, almost all are "imported;" or acquired during travel to endemic areas (Indian subcontinent, Asia, Middle East, and Africa). Of the HEV infections that are symptomatic, the clinical presentations can vary depending on which HEV genotype is causing the infection. With genotype 1 we see acute liver failure (and to a lesser extent with genotype 2), while chronic HEV infections have so far only been observed with genotype 3 and 4 infections[8] (genotypes 3 and 4 cause acute infection as well). Table 2 shows some notable epidemics of HEV that have been reported.

Table 2: Chronological occurrence of major waterborne HEV epidemics and the number of reported cases from the Indian subcontinent, southeast and central Asia including the former Soviet Union[26].

Serial no.	Epidemic region	Country	Year of occurence	No. of reported cases
1	Delhi	India	1955–1956	29 000
2	Kirghiz Republic	Former Soviet Union	1957	10 000
3	Ahmedabad	India	1975–1976	2500
4	Kashmir	India	1978–1982	52 000
5	Hyderabad	India	1989	2000
6	Kanpur	India	1991	79 000
7	Kathmandu	Nepal	1973	10 000
8	Mandalay	Burma	1976–1977	20 000
9	Xinjiang Uighur	Northwest China	1986–1988	119 280
10	Greater Darfur	Sudan	2004	4000

In contrast, genotypes 3 and 4 are transmitted to humans zoonotically from infected animals such as pigs (the major reservoir for HEV), deer, wild boar and shellfish [70,71]. HEV is not only an issue for developing countries, Genotypes 3 and 4 cases are sporadic and occur in industrialized developed countries where they are considered autochthonous, or "locally acquired"[58,72]. These genotypes have a much wider geographical spread and a broad host range[64]. Figure 6 shows the geographical distribution of human HEV disease pattern and human HEV isolates.

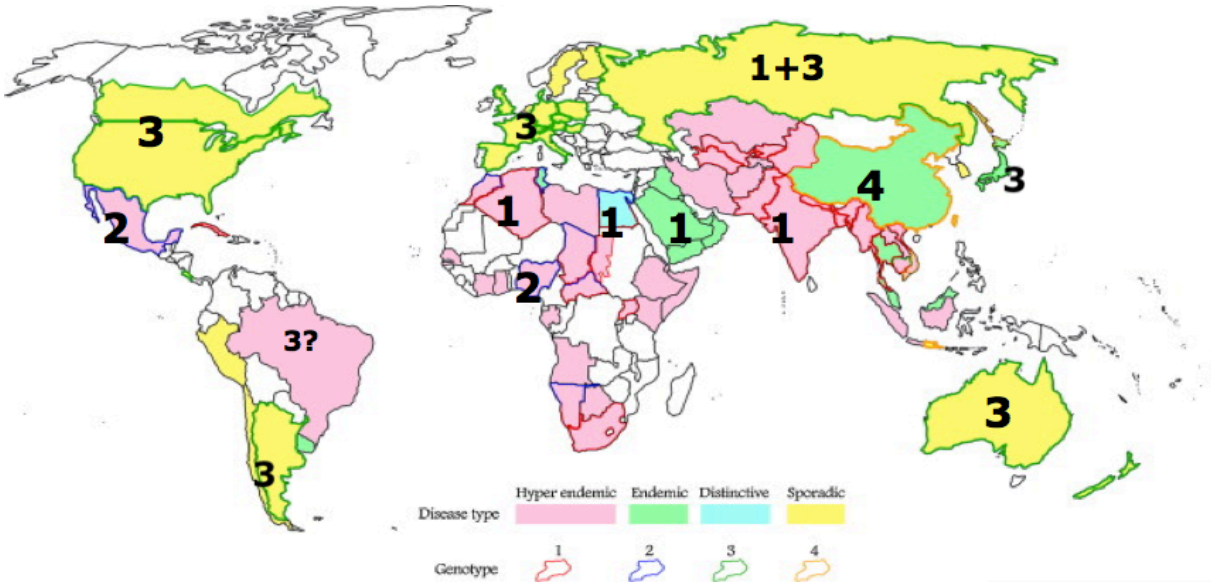

Figure 6: Geographical distribution of human HEV disease pattern and human HEV isolates[11].

In Japan, Europe, and New Zealand there have been some occasional HEV outbreaks through food-borne transmission that have been reported and confirmed. In these cases, affected individuals had consumed undercooked meat that was contaminated with HEV[73–76] . North America has only experienced sporadic cases of foodborne HEV cases thus far. Genotype 3 has been specifically identified as a zoonotic pathogen in developed countries through reports in pig farmers and other individuals who came into close contact with this animal reservoir, or who had consumed raw meat or meat products from pig, deer, and wild boar. HEV RNA had also been detected in liver and sausage sold in supermarkets, and viral sequence extracted from these products had been 99.7-100% similar to sequences of virus taken from HEV-infected patients [77–80]. Documentation has also been made of HEV Genotype 4 infection in both swine and humans in Asia and Europe. All of this evidence supports zoonotic transmission of HEV through consuming undercooked meat [81,82].

A recent study in Canada assessed the national seroprevalence of HEV in Canadian finisher pigs (grower pigs greater than 70kg but not yet ready to be butchered). Farms were recruited using the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) and FoodNet Canada on-farm sampling platforms. They found the overall farm-level prevalence of HEV in finisher pigs to be 34.1% (95% CI: 25.0%, 44.5%) which is comparable to published North American estimates ranging from 25% to 68%[83]. A separate study looked at the prevalence of HEV contaminated pork liver sold in Canadian supermarkets. They detected HEV RNA in 14 of 283 retail livers, for an estimated prevalence of 4.9% (95% CI: 3.0%, 8.1%).[84] This is comparable to a multi-country European study that found an overall HEV seroprevalence of 3.9% in retail pork liver[84]. Indeed, the risk of zoonotic transmission exists in Canada.

1.8 Other Modes of Hepatitis E Virus Transmission

As discussed above, the primary mode of transmission of HEV is fecal-oral through consumption of water contaminated by human feces[14]. Studies have detected HEV genomic RNA sequences in raw and treated sewage water with 100% similarity to that found in patient stool samples. There has also been evidence of fecal contamination in drinking water associated with several HEV epidemics in India following monsoon season[39,85].

Zoonotic transmission due to ingesting contaminated raw or undercooked meat is another major mode of transmission as eluded to earlier. Both domesticated and wild animals have been recognized as potential reservoirs for HEV[65]. Wild boar have been found to have the highest seroprevalence of past or current infection of the wildlife

species studied[70,86] (average seroprevalence of 42.7% among the areas reporting). In addition to swine, HEV has also been detected in other wild game meat, and studies have linked some cases of HEV infection to the ingestion of deer and caribou[87,88]. HEV seroprevalence in deer from countries, primarily in Asia and Europe varies from 2.6% to 34.8% [88–91]. In Europe, roe deer (Capreolus capreolus) and red deer (Cervus elaphus) are considered to play an important role in the maintenance and spread of HEV[88,91,92]. One study found 62.7% of white-tailed deer (Odocoileus virginianus) kept in ranches in northern Mexico had antibodies to HEV [93]. One United States study found no evidence of HEV infection among Sika deer [89]. Currently no data exists on seroprevalence among Canadian wildlife.

Another mode of transmission that is much less common is person-to-person through direct contact. This has been documented in a small number of cases between family members living in the same household. One study reports that HEV transmission through direct contact is responsible for 1% to 2% of cases, whereas for Hepatitis A it is much higher at around 15%[94,95].

Vertical transplacental transmission from mother to fetus during the third trimester of pregnancy has also been described. These affected newborns suffer a high rate of perinatal mortality. There is increased risk of abortions, stillbirths, deaths in newborn babies, and neonatal hypoglycemia and liver injury[6,32]. One study looked at the clinical course of 19 babies born to HEV-infected mothers and found that in 15 (78.9%) of these babies, the virus had been transmitted from the mother (12 were anti-HEV IgM positive, 10 were HEV RNA positive). Of these 15 babies, 7 died during the first week of birth 1 from prematurity, 3 from icteric HEV, 2 from anicteric HEV, and 1 from hyperbilirubinemia[96]. Although there is no evidence for heterosexual transmission of

HEV, a report from Italy showed that 20% of homosexual men had anti-HEV antibodies[35] .

Parental transmission through transfusion of contaminated blood products has recently gained considerable attention. Experiments performed in rhesus monkeys have demonstrated that viral transmission through transfusion of blood plasma from anti-HEV IgM positive and anti-HEV IgG negative blood donors is possible[97]. Patients requiring continuous or multiple blood transfusions or plasma exchange (thalassemia, haemophiliac, leukemia, and thrombocytopenia thrombotic purpura patients for example) are therefore at risk of being infected with HEV from donated blood. Many industrialized countries have now focused on studying the prevalence of blood donors who are HEV positive (see Table 3), as currently no HEV blood donor screening protocolos exist in most countries. Screening involve both serological testing and nucleic acid testing (NAT). Novel kits and techniques have been developed which have increased sensitivity enabling identification of HEV contamination even at very low viral loads (lower limit of detection is 250 IU/ml)[98,99]

Table 3: HEV-RNA positivity among blood donors in various industralized countries

Year of study	Countries	Technique used for detection	No. of tests	Ratio of positive detections
2005	China	Real-time fluorescence RT-PCR	10741	1:1094
2011	England	PCR	42000	1:7000
2011	German	Real-time RT-PCR	18100	1:4525
2011	Sweden	Real-time RT-PCR	95835	1:7986
2011	United States	Real-time RT-PCR	51075	None detected
2011	German	Real-time RT-PCR	16125	1:1241
2011-2012	The Netherlands	Real-time PCR	45415	1:2672
2012-2013	England	RT-PCR	225000	1:2848
2012	France	RT-PCR	53234	1:2218
2013	Spain	Transcription-mediated amplification assay	9998	1:3333

1.9 Seroprevalence of Hepatitis E virus

There is great variability in the seroprevalence of anti-HEV IgG depending on the demographics of the people you sample from, and what part of the world they're from. Seroprevalence in serological surveys have found a low but constant prevalence of 3% in some industrialized countries (relatively high considering the rarity of the disease in these populations), and as high as 70% in some developing countries[100,101].

Frequent contact with animal reservoirs, living or temporarily residing in endemic areas, being male, or consuming liver or undercooked organ meats are all strongly associated with serological HEV positivity[100]. High seroprevalence rates are also found among veterinary and slaughterhouse workers compared to those with no occupational exposure to swine [102]. Those individuals living in swine-dense areas are also more likely to be HEV-positive by serological testing, compared to those who do not live near pig farms[103,104]. Other high-risk groups including patients receiving multiple blood transfusions, injection drug users, and sex-workers are also expected to have higher rates of seropositivity.

In European countries, the diagnosis of acute HEV infection is made in 5%-15% of patients presenting with acute hepatitis for whom hepatitis A-C had been ruled out[105–107]. Most of these patients have traveled to endemic areas, and some have undergone blood transfusion. In addition, when asked about their diet, these patients commonly admit to regularly consuming pork meat. The seroprevalence of anti-HEV IgG in Europe has been increasing over the last decade. Most of these studies performed in the various European countries have been conducted on the general population as well as on blood donors. Prevalence of HEV-RNA positive infection and anti-HEV antibody in blood donors (1%-52% anti-HEV IgG seroprevalence depending on country and study)

strongly suggests that subclinical HEV infection is very prominent[98,108–111]. Table 4 outlines seroprevalence rates in various European populations. Comparison of this seroprevalence data is limited by the quality of the kit used for serological detection, some being less sensitive than others.

Table 4: Hepatitis E seroprevalence in general populations and risk groups in European countries (General populations: BD, blood donors; P, prisoners; CH, children; AD, adult population; ST, students; HW, health workers; C-S, civilians and soldiers; I, Italians; R, refugees; PW, pregnant women; Risk groups (in italics): *SW*, swine workers; *D*, drug users; *FW*, forestry workers; *PT*, patients; *AH*, acute hepatitis; *PV*, pig veterinarians; *NPV*, non-pig veterinarians; *CVHB*, patients with chronic viral hepatitis B; *CLD*, chronic liver disease; *AW*, agricultural workers.) (Adapted from Lapa et al. 2015)[112].

Country	Population	Seroprevalence (%)	Assay
Sweden	BD	9.3	Abbott HEV EIA
	SW	13.0	
Finland	*PT*	11.3	Genelabs
England	BD	16–25	Genelabs
Scotland	BD	4.7	Wantai
Denmark	BD	20.6	In house assay
	P	16.9	In house assay
	D	4.1	Abbott HEV EIA
	SW	50.4	In house assay
Belgium	*PT*	14.0	Biorex diagnostics
Russia	CH	18.2	In house assay
Germany	AD	16.8	Mikrogen
	BD	6.8	Mikrogen
	BD	11.0	Mikrogen
	FW	18.0	Mikrogen
Poland	*PT*	15.9	Adaltis

Country	Population	Seroprevalence (%)	Assay
Moldova	*SW*	51.1	In house assay
Czech Republic	*AH*	5.0	Not done
	AH	27.8	Abbott HEV EIA
The Netherlands	BD	26.7	Wantai
	PV	11.0	Abbott HEV EIA
	NPV	6.0	Abbott HEV EIA
France	BD	52.0	Wantai
	BD	16.6	Genelabs
	BD	3.2	Genelabs
	AH	10.7	Abbott HEV EIA
	FW	31.2	MP Biomedicals
Austria	C-S	14.3	Wantai
Switzerland	BD	4.9	Genelabs
Romania	ST	12.5	Mikrogen
	HW	14.0	Mikrogen
Hungary	*AH*	9.6	HEV Ab, Dia.Pro
Spain	BD	1.08	HEV Ab, Dia.Pro
	BD	3.9	Abbott HEV EIA
	I	5.5	Abbott HEV EIA
	PT	11.4	Mikrogen
Portugal	BD	4	Abbott HEV EIA
Italy	I	3.9	HEV Ab Dia.Pro
	BD	1.3	HEV Ab Dia.Pro
	AH	10.1	Abbott HEV EIA
	AH	20.6	Genelabs
	AH	6.0	HEV Ab Dia.Pro
	AD	5.38	HEV Ab Dia.Pro
Serbia	BD	15.0	In house assay
Albania	*CLD*	36.6	Abbott HEV EIA
Greece	R	4.85	Abbott HEV EIA
	CVHB	5.3	
Turkey	PW	12.6	Virotech
	PW	7.0	Globe Diagnostics
	CH	2.1	HEV Ab Dia.Pro
	CH	8.5	Not done

Country	Population	Seroprevalence (%)	Assay
	AW	34.8	Biyoser S.r.l.

1.10 Study Background

Hepatitis E virus is a major cause of viral hepatitis worldwide, and has a high mortality rate in immunocompromised individuals and in those with underlying liver disease. Recently, HEV seroprevalence studies in industrialized countries have found the rates of HEV among blood donors in industrialized countries to be much higher than expected. Currently no national seroprevalence data exists for Canada.

When looking at the data from studies performed in other countries, it's clear that Canadian blood donation safety and the risk of HEV contaminated plasma pools needs to be investigated to determine the clinical importance of having safeguards in place to protect the recipients. Often the patients are immunocompromised and suffer the greatest morbidity and mortality rate from infection.

The prevalence of HEV infection among wildlife species in North America remains largely undetermined. Mule deer and white-tailed deer are closely related species and the most common wild deer species in Canada. They're an important game animal, and may serve as a potential reservoir for infection. Northern communities rely on game species as a traditional resource for food and cultural practices, particularly the caribou, a keystone herd animal found across Canada.

Certain groups of individuals may be at a much higher risk of HEV infection, in particular, patients receiving multiple blood transfusion and plasma exchange. Studies performed in other countries have also found higher rates of HEV among intravenous drug users as well. Many of these individuals are also infected with HCV and/or HIV, which could potentially further increase their risk of becoming chronically infected with

HEV. Currently no seroprevalence data exists for these potential high-risk groups in Canada.

Another interesting group to assess is the African sex-worker cohorts, most of whom are HIV positive and thus immunocompromised. Due to the proper treatment of HIV patients in Canada, their immune status is much better than HIV patients in Africa who lack adequate treatment. Therefore, HIV infected patients in Africa are more suitable to assess for possible association and impact of HEV co-infection. The cohorts studied previously in areas of Uganda, Kenya, and Tanzania have obtained patient's serum samples from two time points 5 years apart. This allows assessment of seroconversion as well as overall HEV positivity among the cohort of women.

1.11 Objectives

The objective of my project is to determine the seroprevalence of HEV among Canadian blood donors. I'll be testing every fourth sample from 10,000 blood donor samples received from the Canadian Blood Service (CBS) and every second sample from 4,000 samples received from Héma-Québec (HQ). All 14, 000 blood donors will also be tested for HEV RNA (in pools of 48-100). I will also test wild deer samples from species including Mule deer, White-tailed deer, and Caribou for anti-HEV. I will also assess seroprevalence and RNA positivity among hemophiliac patients, leukemia patients, thrombocytic thrombocytopenic purpura (TTP) patients, and intravenous drug users which represent potential high-risk groups for HEV infection and determine if there is a significant difference in infection rates based on seroprevalence and HEV viremia among these groups compared to the general population (approximated by the blood donor group which will likely be similar). Two African sex-worker cohorts will also be

analyzed at two time points 5 years apart to assess seroconversion as well as general HEV seroprevalence and RNA positivity. I used the Wantai Anti-HEV IgG ELISA kit for the human seroprevalence determinations because it has been shown to be the most sensitive assay for detection of this antibody. I tested for NAT using a kit with primers for ORF1 to determine the number of samples that are HEV RNA positive. This will be the first national Canadian study to report seroprevalence of HEV exposure/infection among blood donors. It will also be the first study determining the status of HEV seroprevalence among patients considered at higher risk for acquiring the infection and important wildlife game species in Canada.

1.12 Rationale/Hypotheses

To date, there have been no national studies that have investigated Hepatitis E Virus seroprevalence in Canada. When looking at the data from studies performed in other countries, it's clear that blood safety in Canada needs to be investigated to determine the clinical importance of having safeguards in place to protect the recipients. Often the patients in need of blood transfusion are immunocompromised and suffer the greatest morbidity and mortality rate from infection. I hypothesize that the HEV exposure/infection in Canada as assessed by anti-HEV IgG antibody seroprevalence and HEV RNA among Canadian blood donors will be very low. Prevalence of HEV among wildlife species will also likely be quite low. As well, since the virus is likely not as prominent in Canada compared to other industrialized countries, the rates among the potential high risks groups will be similar. Seroconversion will likely be observed in the African sex-worker cohort over the 5-year period, and seroprevalence rates will likely be very high as this is an HEV endemic region.

2. Materials and Methods

2.1 Sample Collection:

2.1.1 Blood donor samples:

Serum samples were collected by The Canadian Blood Services (CBS) and Héma-Québec (HQ) during 2013-2014. A total of 10,064 and 3,989 serum samples were received from the CBS and HQ respectively. The CBS samples were from the Atlantic, Prairie, and South Central Ontario regions of Canada, while the HQ samples were from various locations within Quebec. All samples were shipped on dry ice and stored at -20°C until tested.

2.1.2 Patients with Hematological malignancies:

The Canadian Bone Marrow Transplantation Group (CBMTG) supplied serum samples (n=54) from patients with some form of leukemia (see Figure 8 for disease distribution) who had multiple blood transfusions (range = 2-101, median =14, total RBC units = 1191). All samples were shipped on dry ice and stored at -20°C until tested.

2.1.3 Samples from Rituximab treated TTP patients who received plasma exchange:

Serum samples were collected from forty patients who had thrombotic thrombocytopenic purpura and who were treated with 375 mg/m^2 of Rituximab once a week for four weeks. Of these forty patients, twenty were refractory and twenty had relapsing TTP. These patients were recruited from four apheresis centers. Plasma exchange was initiated on day 0 and carried out daily for at least 7 to 10 days. During this time they will receive 20-40 liters of pooled plasma. Response to therapy was

measured by platelet count. All but one patient who also got solvent-detergent plasma received either cryosupernatant plasma or Fresh frozen plasma in combination. Blood samples were collected on day 0 and at 1, 12, 24 and 52 weeks for viral studies. All samples were shipped on dry ice and stored at -20°C until tested.

2.1.4 Patients with bleeding disorders:

Serum samples were collected from Canadian hemophiliac patients (n=48) who had received multiple blood transfusions. These samples were taken prior to 1993, when recombinant factor VIII treatment had been introduced. All samples were shipped on dry ice and stored at -20°C until tested.

2.1.5 Intravenous drug user (IDU) samples:

Serum samples (n=268) were collected from intravenous drug users in several health jurisdictions within British Columbia (Fraser Health Authority, Interior Health Authority, Northern Health Authority, Vancouver Island Health Authority). Samples had been stored at -80°C in the National Microbiology Laboratories' long-term storage freezers before testing.

2.1.6 African sex-worker patient serum samples

Archived serum samples taken from cohorts of African sex-worker's that had been previously used in a separate HIV study, were obtained from the NML's long-term storage freezer facility. The first cohort (n=89) was sampled during the period of 1985-1998, the second cohort (n=107) had been sampled during the period from 1999-2008. Some patients provided an additional sample 5 years later. These patients have been

working in the sex-trade for multiple years, and most are HIV positive.

2.1.7 Canadian free-ranging deer samples:

A total of 559 free-ranging deer samples were collected from across Canada. The white-tailed deer serum samples (n = 205) were collected from three different areas and time periods. The Lake Erie region of Ontario (Point Pelee National Park and Long Point National Wildlife area) was sampled from 1990 to 1991, Lunenburg County in Nova Scotia during 2009, and the town of Nipawin, Saskatchewan during 2014. The mule deer serum samples (n = 112) were collected in the municipality of Antelope, Saskatchewan, during 2007 then again in 2012. Caribou serum was also sampled from several locations across Canada. The barren-ground caribou (*Rangifer tarandus groenlandicus*) is a subspecies of the caribou that is found mainly in Nunavut and the Northwest Territories; serum samples (n = 217) were collected from the Bluenose East (2012), Beverly (2009 and 2012) and Bathurst (2011 and 2012) herds. The woodland caribou spans the boreal forest from the Northwest Territories to Labrador; serum samples (n = 97) from the Owl Lake herd, Manitoba, were collected in 2009. The serum samples were obtained from either White-tailed deer collected as part of the annual recreational harvest in Nova Scotia or hunted during regular herd management exercises in Ontario; while the Canadian Wildlife Health Cooperative (CWHC) collected the samples from mule deer and white-tailed deer in Saskatchewan as part of research on chronic wasting disease. Barren-ground and woodland caribou were sampled during the deployment of radio collars for wildlife population monitoring. All samples were kept frozen at -20°C after collection and centrifugation.

2.2 Serological testing of samples:

2.2.1 Human samples:

Serological testing of all human samples were performed using the Wantai Anti-HEV IgG ELISA kits (Wantai, Beijing, China) for the seroprevalence determinations. Some samples (indicated in the results section) were also tested for IgM antibodies using the Wantai Anti-HEV IgM ELISA kits. Assays were performed according to the manufacturer's instruction, using 10µl of serum and 100 ul of diluent per well of the 96-well plate and using the appropriate controls. The ELISA plates were processed and analyzed by the automated Crocodile ELISA miniWorkstation (Titertek-Berthold, Pforzeim, Germany). Cut-off values for determination of IgG or IgM positive samples recommended by the manufacturer state that a sample OD/cut-off value >1.0 is positive, and suggests retesting a sample with a value of 0.9-1.1. We employed a re-testing cut-off value of 1.5 to be even more stringent. This is based on the experience of the diagnostic testing for anti-HEV at NML, samples with OD/cut-off value between 1.0 and 1.5 often could not be confirmed upon retesting in duplicates.

2.2.2 Wildlife samples:

Serological testing of all animal samples were performed using the AccuDiag™ HEV-Ab ELISA (Diagnostic Automation Inc., Woodland Hills, California) for qualitative identification of total antibodies against HEV. This kit utilizes a double antigen sandwich principle, where recombinant HEV antigens (HEV-ag) corresponding to structural proteins ORF-2 of the native virus are pre-coated on the polystyrene microwell strips of the ELISA plate. The serum sample to be tested (50µl) is pipetted into the well and specific antibodies against the HEV-ag will bind to the well. After

washing the plate to remove unbound antibodies, the second recombinant HEV antigen conjugated to Horseradish Peroxidase (HRP) is added to the wells. This antigen, at the second incubation stage, will bind to the second variable domain of the HEV antibodies, if they have been captured by HEV-antigen at first incubation step. This method allows this kit to be used to test serum from any species of animal, because the secondary antibody is not directed against an antibody, but rather the HEV-ag conjugated to it is captured by the test sample if any specific antibodies are present in the serum.

2.3 Detection of Hepatitis E virus RNA:

2.3.1 Pooling of samples and centrifugation:

Blood donor samples for the Canadian HEV seroprevalence study:

All of the blood donor samples for the Canadian HEV seroprevalence study (n=14,053) were combined into pools of 48 or 96 using 100µl of each sample. The pools were then subjected to ultracentrifugation using the Optima L-90K (Beckman Coulter Inc., Brea, CA, USA) to concentrate the potential HEV if present at 150,000g for 90 minutes. The resulting pellet was then resuspended in NucliSENS® easyMAG® (BioMérieux, Marcy l'Etoile, France) lysis buffer to prepare for RNA extraction.

Wildlife seroprevalence study:

All animal samples were tested either individually or in pools: all woodland caribou samples were tested individually, barren-ground caribou were tested in pools of 5 using 100µl of serum per sample, and the rest of the samples were combined into pools ranging from 45 to 73 per pool utilizing 150µl from each sample. The pools were then subjected to ultracentrifugation as above to concentrate the

potential HEV if present. The resulting pellet was then resuspended in 250µl of NucliSENS® easyMAG® lysis buffer to prepare for RNA extraction.

2.3.2 RNA Extraction:

All RNA extractions conducted for this project were performed using the NucliSENS® easyMAG® instrument according to the manufacturer's instructions, with appropriate controls. To begin the extraction, 250µL of lysate was added to extraction buffer (BioMérieux, Marcy l'Etoile, France) in an extraction tray and incubated at room temperature for 10 minutes. Next, 100µL of magnetic silica beads were added to the mixture and mixed thoroughly with the sample solution by pipetting up and down. The extraction was run using the Generic 2.0.1 method. The extraction resulted in the elution of 60µL of RNA solution. RNA extract from this completed step was stored at -80°C until it was used for nucleic acid testing.

2.4 Nucleic acid testing (NAT):

2.4.1 Real-time reverse transcriptase PCR NAT:

Real-time reverse transcriptase PCR was conducted on all samples using the RealStar® HEV RT-PCR kit (Altona Diagnostics, Hamburg, Germany) with primers for an ORF 1 consensus sequence of the HEV RNA genome as per manufacturer's instructions. The test utilizes real-time RT-PCR technology, using reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labeled with fluorescent reporter and quencher dyes.

Probes specific for HEV RNA are labelled with the fluorophore FAM. The probe specific for the target of the Internal Control (IC) is labelled with the fluorophore JOE. Using probes linked to distinguishable dyes enables the parallel detection of HEV specific RNA and Internal Control in the corresponding detector channels of the real-time PCR instrument, thus allowing us to make sure the test was valid by proving there were no potential inhibitors present in the RNA extract. Positive control used for this assay was serum from a confirmed high titre HEV-positive patient.

2.4.2 Hemi-nested PCR

Screening was done by hemi-nested RT-PCR using broadly reactive oligonucleotides targeting viral RNA-dependent RNA polymerase region of ORF1 according to author's instructions [66]. The assay was designed to amplify all members of the family Hepeviridae available in GenBank. The assay sensitivity was determined to be on the order of 10 copies per reaction by using a quantified in vitro transcript (HEV genotype 3).

2.4.3 Detection of Amplicon

The amplified PCR product was run and visualized on the QIAxcel multicapillary electrophoresis system (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions.

3. Results:

3.1 Canadian Hepatitis E Seroprevalence Study

To determine the overall seroprevalence of HEV among the general Canadian population, we analyzed a total of 14,053 blood donor samples from Canadian blood banks. Although there are requirements that guide who can donate blood (individuals are excluded based on certain lifestyle factors, recent travel, or medical/sexual history) this sample group still provides a relatively accurate picture of the current status of HEV prevalence in the general Canadian population. Serological testing for anti-HEV IgG antibody was performed on every fifth sample received from CBS (n=2,160) and every second sample from HQ (n=1,947) and seroprevalence was determined. There were 110 (5.09%) positive samples from CBS, and 130 (6.68%) positive samples from HQ. This gives a total of 4,107 samples tested from across Canada, and 240 (5.84%) samples testing positive. All IgG positive serum samples were then also tested for anti-HEV IgM as this antibody is indicative of a recent or current infection. Of the 240 IgG Positive samples, only 9 (3.75%) of these were also IgM positive. This is 0.064% of the entire blood donor cohort we tested.

Table 5: Breakdown by Canadian Blood Service Centre of blood donors eligible to participate in the study and participation rates (Canadian Blood Services)

CENTRE	Number of donors during the study period	Number of donors at clinics randomly selected for testing	Number of donors tested	Percentage of donors tested during the study period
Halifax	2,778	940	542	19.5
New Brunswick	9,854	2,775	1,223	12.4
Hamilton	21,992	2,718	1,868	8.4
London	16,090	3,079	2,088	13.0
Toronto	20,932	4,383	2,566	12.3
Winnipeg	12,313	2,730	1,775	14.4
TOTAL	83,959	16,625	10,062	12.0

Table 6: Seroprevalence among Canadian Blood donors by geographical region with associated average sample to cut-off ratios

	Seroprevalence		**Sample/Cutoff Ratio**		
Region	**#**	**%**	**Average**	**Range**	**St. Dev.**
Atlantic	6/334	1.80	6.86	1.8-14.4	5.19
Prairies	18/356	5.06	6.95	1.5-17.3	4.93
S. Central Ontario	86/1470	5.85	7.17	1.12-18.9	4.84
Quebec	130/1947	6.68	8.02	1.11-21.23	5.69

The seroprevalence did not differ significantly by region; however, the seroprevalence rate in the maritime region was three-fold less than the other regions surveyed (not significant). The sample/cutoff ratio indicates the relative level of anti-

HEV IgG antibody. A higher number indicates a strong positive and suggests a more recent infection. The cutoff value is the OD value above which we call a sample positive for HEV antibody. There was not a significant difference in strong positives between regions, as outline by Table 7.

Table 7: Proportion of anti-HEV IgG positive samples that were strong positives indicated by a test sample/cutoff ratio greater than 10, by geographic region

	Strong IgG Positive (Sample/Cutoff >10)	
Region	#	%
Antlantic	2/6	33.33
Prairies	5/17	29.41
S. Central Ontario	26/85	30.59
Quebec	53/129	41.08

Table 8: HEV antibody positive test results, by sex and age n=2150 (Canadian Blood Services).

	Positive	**Negative**	**% Positive**
Sex			
Female	48	945	**4.83**
Male	62	1095	**5.36**
Age			
Under 29	7	474	**1.46**

30-39	4	276	**1.43**
40-49	16	395	**3.89**
50+	83	895	**8.49**
Total	**110**	**2160**	**5.09%**

Table 9: Seroprevalence by age and gender group for the HQ donor group (Héma-Québec)

	Positive	**Negative**	**% Positive**
Sex			
Male	84	975	**7.93**
Female	47	846	**5.26**
Age			
18-29	8	447	**1.76**
30-39	6	248	**2.36**
40-49	14	332	**4.05**
50-59	41	486	**7.78**
≥60	62	308	**16.76**
Total	131	1821	**6.71**

Chi-square analysis of age group stratification shows that the 40-49 and 50+ age groups are significantly more likely to be seropositive compared to the under 29 and 30-39 age groups in both the CBS and HQ sample groups (p=<0.0001). Univariate analysis by gender showing males are significantly more likely to be seropositive than females across Canada (p=0.035).

Table 10: % Prevalence Vs. Sample/Cut-off value for Héma-Québec donor group.

Age	**S/CO 1-3.99**	**S/CO ≥ 4**
18-29	**4 (0.88%)**	**4 (0.88%)**
30-39	**3 (1.18%)**	**3 (1.18%)**
40-49	**5 (1.45%)**	**9 (2.60%)**
50-59	**13 (2.47%)**	**28 (5.31%)**
≥60	**21 (6.49%)**	**38 (10.27%)**

Table 10 Indicates the number of patients with a low sample/cutoff ratio as well as the number of patients with a high sample/cutoff ratio. A higher sample/cutoff ratio indicates a more recent exposure/infection. Patients above 50 are significantly more likely to have had a more recent exposure/infection ($p=<0.0001$) and/or immunity booster from continuous exposure.

3.2 Assessment of HEV seroprevalence among high-risk groups, and analysis of associated risk factors:

Certain groups of individuals, due to medical treatment or lifestyle choices, are hypothetically at an increased risk of acquiring the Hepatitis E virus. We expect that

these groups will demonstrate an increased seroprevalence compared to the blood donor group. High risk groups we assessed in this study are as follows: individuals receiving multiple blood transfusions (patients with hematologic malignancies or coagulation defects), those undergoing plasma exchange or receiving other plasma products (TTP patients), people who inject drugs (PWID), and those working in the sex trade that are HIV-positive.

3.2.1 Hepatitis E seroprevalence among people who inject drugs in the Vancouver area:

To determine whether PWID, a group at high-risk for HEV exposure, experience higher seroprevalence rates than the blood donor cohort, we tested a total of 268 patient samples from the Vancouver area collected between 2011 and 2013 that are confirmed PWID. Of these 268 patients, 18 (6.72%) tested positive for anti-HEV IgG. These patients ranged in age from 26-71 years of age, with the median age being 45 (13 samples did not provide age data). Of these patients, 59 were female, 190 were male, and there were 19 samples that did not have gender data attached. There were 1 of 59 (1.70%) female patients and 15 out of 190 (7.89%) male patients testing positive for anti-HEV IgG, although this difference was not statistically significant.

There were 46 HIV-positive PWID's within this cohort, and 2 (4.35%) of these individuals were also HEV-positive. HCV serology had not been done on either one of these HIV-positive HEV-positive patients. One of the HIV-positive HEV-positive patients was male age 55; the other patient was female age 42. Comparing this to the 205 HIV-negative IDU's within this cohort, 15 (7.32%) were HEV-positive. The difference in HEV IgG seropositivity by HIV status was not significant in this group. There were 17 patients who had not been tested for HIV. Of 158 HCV positive patients, 8 (5.06%) were

HEV positive. There were 110 patients who were not tested for anti-HCV IgG. This data is summarized in Table 12.

Comparing HEV IgG seropositivity by age, there were 247 patients 30 or older of which 17 were anti-HEV IgG positive (6.88%) vs. 0 anti-HEV IgG positive out of 8 under 30 (0.00%). Of 187 patients 40 or older, 17 were anti-HEV IgG positive (9.09%) vs. 0 anti-HEV IgG positive out of 68 under 40 (0.00%). Of 75 patients 50 or older 12 were anti-HEV IgG positive (16.0%) vs. 5 anti-HEV IgG positive out of 180 under 50 (2.78%). The increase in seroprevalence with age is statistically significant (p=0.001 using fisher's exact test) (see Table 12). The cohort of anti-HEV IgG positive individuals in this study ranged in age from 41 to 71, with the median age being 54 years of age. There were proportionately more males than females that tested positive for anti-HEV IgG, however this difference was not statistically significant. All anti-HEV IgG positive samples were tested for anti-HEV IgM and all were found to be negative.

Table 11: Demographic data comparing HEV infection by gender and age category for IDU and Blood donors

Gender	Age Group*	# anti-HEV IgG Positive/ Total Tested	Seroprevalence (%)	95% Confidence Interval (%)
		PWID	PWID	PWID
Male	-	15/190	7.89	4.08 to 11.70
Female	-	1/59	1.70	-1.6 to 4.98
	<30	0/8	0.00	-0.69 to 0.69
	>30	17/247	6.88	3.72 to 10.04
	>40	17/187	9.09	4.97 to 13.21
	>50	12/75	16.00	7.7 to 24.3

3.2.2 Hepatitis E seroprevalence among sex workers in Africa:

A total of 196 individuals were tested for anti-HEV IgG at least once during the study period between the two cohorts (89 from cohort 1: from years 1985-1995, 107 from cohort 2: years 1999-2008). Of the 196 individuals tested, 156 were tested a second time approximately 5 years later. A total of 60/196 (30.61%) individuals tested positive for anti-HEV IgG at least once, 49 were form Cohort 1 (55.06%) and 11 were from Cohort 2 (10.28%). This difference is statistically significant ($p<0.001$). Of 156 total individuals tested at two points, 15 individuals seroconverted (13 of these were HIV-positive), and 10 individuals lost their anti-HEV IgG seropositivity (all 10 were HIV-positive).

There were 121 HIV-positive individuals between the 2 cohorts, of which 55 (45.45%) were also serologically positive for HEV. Of the 75 individuals negative for HIV between the 2 cohorts, 5 (6.675) were serologically positive for HEV. This difference was significant ($p<0.001$). However, the comparison cannot be made within each cohort separately due to the small sample size.

From the 89 individuals comprising cohort 1; 4/6 (66.66%) of Kenyans, 47/89 Tanzanians (52.81%), and 0/1 (0.00%) Ugandans tested positive for anti-HEV IgG at least once. From the 107 individuals compromising cohort 2, 12/89 (13.48%) of Kenyans and 1/18 Tanzanians (5.55%) tested positive for anti-HEV IgG at least once. Although they have different nationalities, they all live and practice their trade in Nairobi. We have limited data about the immune system status based on CD4 count (n=21) and there not a difference in the seroprevalence based on this.

3.2.3 Hepatitis E seroprevalence among leukemia patients who received multiple blood transfusions:

Another group at high-risk of HEV exposure due to both illness and frequent blood transfusions, are leukemia patients. We tested a cohort of patients (n=54) with various forms of leukemia (Figure 8) using serum samples provided by the CBMTG. The HEV-IgG seroprevalence was found to be 11.11% (6/54). All patients were also tested for anti-HEV IgM, and 2 of the 54 patients were found to be positive (3.70%). None were positive for HEV RNA by PCR.

3.2.4 Hepatitis E study in Rituximab treated Thrombotic Thrombocytopenic Purpura (TTP) patients who received plasma exchange:

Thrombotic Thrombocytopenic Purpura patients who received large volumes of plasma exchange are also expected to have higher rates of HEV exposure than the general population and blood donor group. There was no HEV seroconversion observed in the patients (n=38) after 52 weeks of treatment. The HEV seroprevalence among this patient cohort was 21.05% (8/38, see Table 13). There was one patient who became weakly IgM positive at week 24. This patient was negative for HEV RNA. In four of the cases anti-HEV persisted throughout the observation period, and in another case anti-HEV IgG was lost. In one case, there was a boost in the anti-HEV IgG antibody titre. All patients were followed for one year after initiation of treatment and despite being immunosuppressed by the Rituximab, did not acquire HEV.

Table 12: HEV seroprevalence in high risk groups and Canadian blood donors.

Risk Group	Subgroup	# anti-HEV IgG+/ Total Tested	Seroprevalence (%)	95% CI(%)
Canadian Blood Donors	**-**	**240/4107**	**5.84**	**5.12 to 6.56**
PWID	**Total**	**18/268**	**6.72**	**5.12 to 6.56**
PWID	**HIV+**	**2/46**	**4.35**	**-1.08 to 9.88**
PWID	**HCV+**	**8/158**	**5.06**	**-0.21 to 9.91**
Leukemia patients	**-**	**6/54**	**11.11**	**2.73 to 19.49**
TTP patients*	**-**	**8/38**	**21.05***	**8.09 to 34.01**
Hemophiliac patients	**-**	**2/48**	**4.17**	**-1.49 to 9.83**

*Indicates statistical significance ($p<0.05$)

3.2.5 Hepatitis E seroprevalence among Canadian Haemophiliac patients who received multiple blood transfusions:

Hemophiliac patients receiving multiple blood transfusions represent the fourth high-risk group of individuals studied in this project. The patients compromising this cohort are from various locations in Canada, and were collected prior to the use of recombinant Factor VIII proteins. The HEV seroprevalence among this cohort (n=48) was found to be 4.17% (2/48). There were no anti-HEV IgM positive individuals (Table 12).

3.3 Canadian free-ranging deer seroprevalence study:

Free-ranging deer in Canada are commonly hunted and serve as an important food source for many people in Canada, particularly the First Nations groups in the northern regions. Inuit frequently eat their meat raw or fresh frozen which increases the risk of infection should the animal be carrying the virus. Deer often come into close contact with livestock, including pigs, but also wild swine species such as the wild boar. This could represent another potential reservoir for infection. To explore the level of previous exposure of deer to the virus, HEV seroprevalence was determined in several species of deer from across Canada. The data for this is illustrated in Figure 7 and Table 12. A total of 534 deer were sampled from across Canada, and 30 (5.62%) tested positive for HEV-IgG.

Overall, white-tailed deer were found to have the highest seroprevalence rate of the species investigated, with 18 out of 205 animals tested positive (8.8%). Mule deer followed with 5 out of 112 (4.5%) testing positive, and caribou had the least seroprevalence rate with 7 out of 217 animals testing positive (3.2%).

Seroprevalence rates among the different herds of white-tailed deer we sampled from Nova Scotia, Ontario and Saskatchewan ranged from 4.9%-11.5%; these differences were not statistically significant. Significantly more male deer were positive for anti-HEV than females in Nova Scotia ($P = 0.01$; Fisher's exact test).

Figure 7: Free-range deer sampling sites in Canada. Local seroprevalence (%) is marked next to the schematic images of white-tailed deer, mule deer and two subspecies of caribou. Satellite locations of Bluenose-East, Bathurst and Beverly barren-ground caribou herds can be found on (Http://www.enr.gov.nt.ca/sites/default/files/15.4mapofbarren-groundcaribouherds.Jpg)

Table 13: Hepatitis E virus seroprevalence in white-tailed deer, mule deer and caribou in different geographic locations in Canada

Deer species/Region	No. seropositive / No. samples	Seroprevalence% (95%CI)
A. White-tailed deer (total)	**18/205**	**8.8 (5.5 -13.5)**
1. Lake Erie (total)	8/106	7.5 (2.5-12.6)
a) Point Pelee National Park	3/61	4.9 (-0.5 -10.3)
b) Long Point Provincial Park	5/45	11.1 (1.93 - 20.3)
2. Province of Nova Scotia (total)	7/73	9.6 (4.4 - 18.8)
a) Italy Cross community	4/36	11.1 (3.8 - 25.9)
b) Lunenburg	3/37	8.1 (2.1-22.0)
3. Municipality of Antelope, Saskatchewan	3/26	11.5 (3.2 -29.8)
B. Mule deer (total)	**5/112**	**4.5 (1.7-10.3)**
1. City of Nipawin, Saskatchewan		
a) collected in 2007	0/47	0
b) collected in 2012	5/65	7.7 (2.9-17.2)
C. Caribou (total)	**7/217**	**3.2 (1.6 -6.5)**
1. Barrenground caribou (total)	2/120	1.7 (0.08 -6.2)
a) Bluenose East herd	1/50	2 (0.01 - 11.5)
b) Bathurst Herd	0/23	0
c) Beverley herd	1/47	2.1 (0.08 -12.4)
2. Woodland caribou Owl Lake herd, Manitoba	5/97	5.15 (2.2 - 11.5)

Seroprevalence did not differ significantly based on the year of sample collection or age. Hepatitis E virus seroprevalence also did not differ significantly between mule and white-tailed deer in Saskatchewan or among white-tailed deer from Ontario and Nova Scotia. Anti-HEV was not detected in Saskatchewan in 2007, but was present in 7.7% of mule deer sampled at the same location in 2012.

Woodland caribou were three times more likely to have anti-HEV compared to the barren-ground caribou although the difference was not statistically significant. HEV seroprevalence in all caribou was significantly less than that of the white-tailed deer (P= 0.02). Hepatitis E virus RNA was not detected in any of the samples by RT-PCR.

4. Discussion:

The seroprevalence of hepatitis E reported in many industrialized countries has been much higher than predicted, considering the relatively rarity of the disease. Although HEV is often self-limited in healthy individuals, the clinical course in patients with underlying liver disease and those who are immunocompromised can be very severe, leading to acute liver failure and even death. Patients receiving blood transfusions are often immunocompromised, necessitating an investigation aimed at assessing the risk of blood-borne HEV-transmission to ensure patient safety. This study was the first national Canadian survey investigating the prevalence of HEV among Canadian blood donors. Overall, the seroprevalence was quite low even when compared to similar European countries. We found 5.84% of the 4,107 blood donors tested for anti-HEV IgG to be positive. One U.S. study reported 18%[102] while in another more recent study it was 9.5%[113]. In England, a study found that the seroprevalence of HEV among blood donors to be 16% [114], Denmark 20.6% [115] , France16.6%-52.0% depending on region surveyed [112,116], Switzerland was 4.9% [117] , Scotland 4.7% [118] , and in the Netherlands it was 2.0%[112]. Comparison of all these seroprevalence data has its limitation in that different HEV antibody assays may produce different results. Some studies are performed with kits that are more sensitive than others, which create issues when comparing studies from different countries or from studies performed in the same country using different kits. This study used the Wantai Anti-HEV IgG ELISA kit for the human seroprevalence, which has been shown to be the most sensitive assay for detection of this antibody [40] . Our data strongly suggests that HEV is not as prevalent in Canada as in other industrialized countries.

The seroprevalence by Canadian region outlined in Table 6 shows that it is fairly similar across Canada, with the exception of the Maritime region. This region experienced an approximately three-fold lower seroprevalence than the other regions, although this difference was not statistically significant. It is not clear why there is this tendency. There were no significant differences in the proportion of samples with high antibody levels based on S/CO values by region (Table 7). A strong IgG positive sample indicates a more recent infection, and could mean that this region has experienced a more recent infection or continuous low level HEV exposure. HEV seroprevalence significantly increased with age in this study, which agrees with studies performed in several other countries [109,112,116]. Males also experienced significantly higher seroprevalence rates than females which also agrees with other studies [114,118].

Another avenue that has been investigated is the prevalence of HEV-RNA-positivity among blood donors using Nucleic Acid Testing (NAT). Real-time PCR studies has revealed that indeed there are HEV positive donors who did not present with Hepatitis E symptoms at the time of donation. In England, 6 of 880 (0.7%) mini-pools (48 donors per pool) were positive for HEV RNA [109], corresponding to a minimum prevalence of 0.014%. Recently, another English study retrospectively screened 225,000 blood donor samples and found 79 positive with HEV RNA, giving an RNA prevalence of 1:2848 (0.0351%) in this population[119]. Similar studies investigating plasma donations from Germany, Sweden, and the United States found positive rates of 1:7986 (0.0125%) and 1:4525 (0.021%) for Swedish and German donations respectively [108]. One study performed in the U.S. study failed to detect any positives[108], however; a 2016 study also performed in the U.S. found 2 PCR positive samples out of 18,829 samples tested (0.0106%)[113]. A study done in Japan using donors who had

elevated alanine aminotransferase (ALT of ≥61 IU/l), who are likely to have an ongoing HEV infection, found positive HEV RNA results of 0.3% [120]. Of the 14,053 blood donor samples included in this study, there were none that tested positive for HEV RNA by real-time RT-PCR or our in-house hemi-nested PCR. This further substantiates our claim that HEV is not as prominent in Canada as in other industrialized countries. A possible limitation in our study is the method used to pool samples before centrifugation, which lowers the sensitivity of the screening method from 20 IU/ml to 250IU/ml. However, the same or similar approach used in other studies still came found viremic blood donors in European countries

There are several risk factors that can make it more probable for an individual to acquire the virus. Location (endemic areas), Diet (consuming contaminated raw or undercooked meat), frequent contact with animal reservoirs, gender (being male), being immunosuppressed/immunocompromised are all risk factors that increase the chance of infection. In addition, persons whom due to medical illness require multiple transfusions to treat a hematological disorder or malignancy may acquire the virus through a contaminated blood donation. Some illnesses require huge volumes of plasmapheresis to treat their condition. Again, these patients are subjected to the risk of being infected if the plasma pool is contaminated. Intravenous drug use is another risk factor for acquiring HEV through parenteral transmission, and possible immunosuppression due to higher rates of HCV and/or HIV infection within this population. Last, prostitution is another risk factor due to the high rate of HIV infection. In this study, we assessed several of these risk factors by analyzing cohort data from representative populations.

Patients receiving frequent blood transfusions are considered to be at high-risk for HEV, and indeed there have been reports of post-transfusion Hepatitis resulting from donors who were HEV RNA positive but asymptomatic at the time of donation[1,121–126]. A recent study performed in England that retrospectively screened 225,000 blood donor samples, found 79 samples that tested positive for HEV RNA (all were Genotype 3). Recipients, who received any blood components from these donations, were identified and the outcome of exposure was ascertained. The 79 donations had been used to prepare 129 blood components, 62 of which had been transfused before identification of the infected donation. Follow-up of 43 recipients showed 18 (42%) had evidence of infection (seroconversion).[119]

Individuals with hematological disorders or malignancies (hemophiliacs and leukemia patients) require multiple blood transfusions over their lifetime, and if the blood supply is contaminated with HEV, this group could suffer a transfusion-transmitted infection, and thus may have higher rates of HEV than in the general population. However, our study failed to find any difference in infection rates among hemophiliacs, as only 4.17% of the individuals in our study were HEV IgG positive. These patient samples were collected before 1993 prior to the use of recombinant factor VIII, meaning they were treated with plasma products. One limitation in this study is the small sample size (n=48).

Leukemia patients are immunosuppressed due to the chemotherapy treatments they receive and this further increases their vulnerability to transfusion associated HEV infection. There were 11.11% of leukemia patients that tested positive for anti-HEV IgG but this difference was not statistically significant when compared with the blood donor cohort. All patients were also tested for anti-HEV IgM, and two leukemia patients

(3.70%) were positive. All IgG/IgM positive samples were tested for HEV RNA by PCR, all were negative. A small sample size (n=54) could be a limitation in this study, however combined with the group of hemophiliacs which has the same risk factor, the total number is 102. This lack of increased seroprevalence, despite frequent transfusion is strong evidence that the Canadian blood supply is safe and carries a very low risk for transfusion-acquired infection.

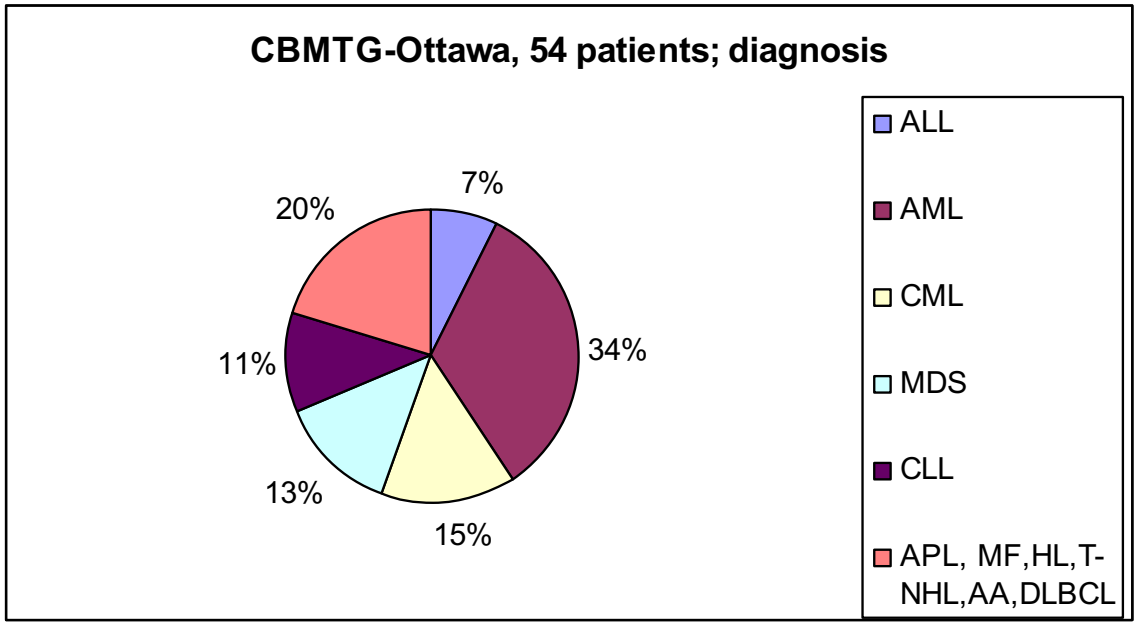

Figure 8: Proportion of the types of Leukemia among the Leukemia cohort supplied by the CBMTG.

Thrombotic thrombocytopenic purpura (TTP) is a severe blood disorder characterised by extensive clots (platelet-rich thrombi) within the blood vessels causing thrombocytopenia, hemolytic anemia and neurological and renal impairment. Plasma exchange (1.5 volumes every day for the first 3 days) is considered the standard

therapy for TTP. It removes antibodies (proteins) from the blood that damage the ADAMTS13 enzyme. Plasma exchange also replaces the ADAMTS13 enzyme. During plasma exchange, an IV needle or tube is placed in a vein in the patient's arm to remove blood. The blood goes through a cell separator, which removes plasma from the blood. The non-plasma part of the blood is saved, and donated plasma is added to it. This donated plasma is taken from a pool of up to 2500 donors. It's apparent that these patients are especially vulnerable to potential transmission of transfusion associated HEV infection due to the large volumes of plasma. In our study, thirty-eight TTP patients received plasma exchange treatment (20-40 litres of plasma per patient); seventeen received SD-plasma (pooled plasma treated with solvent detergent), nineteen were treated with cryosupernatant plasma (concentrated pooled plasma) and two with fresh frozen plasma and Pentaspan or albumin. None of the patients demonstrated any clinical signs of viral hepatitis during the 6-month period of observation. Three samples were collected from TTP patients at time 0, 1 and 6 months' post-treatment and tested for anti-HEV antibodies. Patients with HEV seroconversion were also tested for viremia by PCR. We found a significantly higher seroprevalence of HEV IgG among the TTP patients (21.05%) compared to the blood donor group (5.84%). However, anti-HEV IgG may be acquired passively because of the large volumes of plasma exchange and/or the intravenous gammaglobulin used for treatment.

TTP are also treated with Rituximab for relapsed refractory TTP. Rituximab is a monoclonal antibody against CD20, found primarily on B cells. Rituximab binds to the B cells and causes its destruction. Patients therefore have reduced immune response and are immunosuppressed. Patients were given Rituximab at a dose of 375 mg/m^2 at week

3 and 4. Blood samples were collected at time point 0,4,24, and 52 weeks. We observed no HEV seroconversion over the 52 weeks, and no anti-HEV IgM findings. In four cases anti-HEV persisted throughout the observation period, and in one case anti-HEV IgG was lost (possible due to the Rituximab's effect on the B cells). In one case there was a boost of anti-HEV IgG (this may be from exposure). Despite of the durable immunosuppression induced by the Rituximab, no patients acquired HEV. Again, this provides additional evidence of adequate blood safety in Canada, and very low risk for acquiring HEV through blood products. This data is in line with that from the cohort of hemophiliac patients, and cohort of leukemia patients suggests that we do not require any specific screening protocol for HEV in the current blood/plasma donation guidelines.

Studies performed in low endemicity countries including Brazil, Italy, Iran and Switzerland have reported significantly higher rates of HEV exposure among intravenous drug users[127–129]. However similar studies performed in Denmark, France and the U.S. rejected this association, and found similar seroprevalence rates in the general population as in this risk factor group [130,131]. These individuals commonly share and reuse needles, and this can lead to parental transmission of the virus from an infected individual. Higher rates among this population may also be due poor hygiene (fecal-oral transmission) or may also be facilitated by sexual practices (direct contact). The water used to dissolve the drug before injection may also be contaminated with HEV, representing another source of contamination. In addition, this population has higher rates of HCV and HIV infection. Some studies have reported an association of HEV infection with other viruses [98,132–135]. This study found no significant difference in seroprevalence between PWID's and the blood donor population (6.72% vs. 5.84%

respectively). There was also no significant difference among individuals that were HIV positive or HCV positive compared to the blood donor population (4.35% and 5.06% respectively), in fact the rates were slightly lower. These results may be accounted for due to the very low prevalence of HEV in the general Canadian population, so even though these individuals may be at a higher risk, there is still a relative absence of the virus in circulation and so they still do not acquire it. Male PWID's experienced higher HEV infection rate, though this was not significant. As in the blood donor group, seroprevalence increased with age. None of the positive individuals was a recent infection, as none were positive for anti-HEV IgM antibody.

We tested the Kenyan sex-worker cohorts for anti-HEV IgG in order to assess whether there is a significant difference between individuals who are HIV-positive and those who are HIV-negative. A total of 196 individuals were tested for anti-HEV IgG at least once during the study period. Of these 196 individuals tested, 121 were HIV positive and 75 were HIV-negative. There were significantly more HEV-positive individuals in the HIV-positive group compared to the HIV-negative group (45.45% vs. 6.75% respectively, p= <0.0001). However, the comparison cannot be made within each cohort separately due to the small sample size. One of the limitations of the Chi-square 2x2 contingency table test is that all the expected values must be at minimum "5", and due to the small sample size of each cohort, this test function was not fulfilled. The difference by HIV status is likely due to the immunosuppression caused by the concomitant HIV-infection, or due to increase risk of other epidemiological confounders. Since HEV is endemic in this area, we suspect this is why we are seeing this significant difference (compared to our Canadian PWID study, where it is not endemic). It's interesting to note that in the HIV-negative group, the seroprevalence is

quite low and even comparable to the rates of most industrialized countries. We observed an approximate seven-fold increase in the HIV-positive group. This is evidence that having HIV-infection maybe be a significant risk factor for HEV-infection. Most other studies have reported increased seroprevalence of HEV in those infected with HIV compared to those who are HIV-negative, granted there is a wide variation between studies[136].

There were significantly less seropositive individuals in cohort 2 (10.28%, sampled during years 1999-2008) than in cohort 1 (55.06%, sampled during years 1985-1995). This decrease could be due to improvements in hygiene or socioeconomic conditions; but could also be due to a lower proportion of HIV-positive individuals in cohort 2 compared to cohort 1 (30.84% versus 96.63%). Further study is needed to confirm this association.

The wildlife study we conducted, which utilizes the species-independent double-antigen sandwich method (sensitivity 99.8%, specificity 99.6%), circumvents some of the problems faced by previous researchers aiming to detect HEV antibodies in different animal species, and has been used successfully for wild boar and red deer [91]. Using this method, there is no need to modify commercial HEV immunoassays, or develop an in-house assay using recombinant HEV proteins for indirect sandwich ELISA. Using this species-independent format, you can avoid the specificity reducing cross-reactivity that is inherent in the anti-species-specific capture antibodies and conjugate system. Although this system has enhanced sensitivity to detect anti-HEV antibody, one limitation is the inability to distinguish class-specific antibodies such as anti-HEV IgM allowing the detection of a more recent infection.

This study demonstrates serological evidence proving that deer in Canada have been exposed to HEV or HEV-related viruses. White-tailed deer are very abundant in Canada, and a commonly hunted game animal used as a meat source. With furthering human development, cutting of forested areas and clearing brush land; there is diminishing deer habitat bringing people and their farmlands closer to the deer where they may contact domestic pigs. In addition to domestic swine, wild boar has the potential to become a reservoir for HEV. Higher seroprevalence has been reported in areas with high wild boar density [86]. There is no transmission link between wild boar and deer in Canada suggested by our data. The observed seroprevalence in Nova Scotia and the two Lake Erie sites in Ontario where wild boar are absent were similar to that observed in Saskatchewan where wild boar and pig farms are abundant. With increasing contact between deer of the same species there comes an increased spatial risk of exposure to zoonotic pathogens such as HEV.

The barren-ground caribou samples used in this study were taken from three different herds that range seasonally between the tundra and taiga of northern Canada. Very few of these animals (1.7%) tested positive for anti-HEV. Documentation shows that there is limited overlap between the habitats of the barren-ground species with other species that may harbor HEV. This lowers the chance of exposure to other animals that could be a reservoir of infection. The Woodland caribou in Manitoba had a seroprevalence rate that was three times higher, however, this difference was not statistically significant. Woodland caribou inhabit more southern latitudes where they may encounter several different other species of animal, including white-tailed deer which were found to have similar prevalence of anti-HEV antibodies. In northern Inuit communities and culture, the barren-ground caribou is an important food source that is

regularly consumed; often dried, smoked, age-frozen or otherwise eaten raw. Although this increases the likelihood of becoming infected if the meat is contaminated, the observed low seroprevalence tells us that the potential for foodborne exposure is likely very low. A recent serological survey reported that only 3% of the Inuit population in Canada had evidence of past HEV infection [137]. Although HEV viremia has frequently been detected in wild boar and deer in Europe, it was not found in this study. The lack of viremia in the three species of deer included in this study indicates a less prominent risk of zoonotic transmission compared to other countries.

5. Conclusion

Hepatitis E Virus remains a substantial cause of morbidity and mortality worldwide, predominantly in the developing areas. Our study found that currently, HEV is not prevalent in Canada and poses very little threat to national blood supply. The seroprevelence in the blood donor cohort was less than in similar studies performed by other industrialized countries, and we did not detect a single donor sample positive for HEV RNA out of the 14,053 tested. The blood donor cohort is likely very representative of the general Canadian population.

We analyzed several cohorts with a risk factor that puts them at high-risk for HEV infection. The cohort consisting of hemophiliacs and leukemia patients who have received multiple blood transfusions, did not display increased seroprevalence compared to the blood donor group. This is evidence of low HEV prevalence, and also low risk to the blood supply. TTP patients receiving large volumes of plasma by plasmapheresis did in fact have significantly higher seroprevalence than the donor group, but it possible that this could be due to passive antibody transfer during the treatment. TTP patients are also treated with Rituximab, a powerful immunosuppressant. Despite their lowered immune function, none of these patients developed clinical signs or laboratory markers of acute infection. The intravenous drug user cohort, many of whom were also HIV-positive and/or HCV-positive, also showed no significant difference. We did not see a difference with HIV status in this group either. All of this evidence suggests that currently HEV is not common in Canada. When comparing HIV status with HEV seropositivity in an endemic area (Kenya and surrounding areas of Africa), our study found a significant increase in HIV-positive individuals suggesting this group is at high-risk for HEV infection. Further study is

needed to confirm this association.

Also assessed in this study was the seroprevalence in particular wildlife species that are commonly hunted game animals. White-tailed deer displayed the highest seroprevalence but no viremia and their potential of being a reservoir for infection should be investigated further. Caribou is commonly consumed raw in northern cultures, but the caribou seropositivity in nearby herds was almost non-existent and none of the animals were viremic indicating the zoonotic risk to humans is negligible.

Current blood donor screening protocols should be sufficient, as it appears that HEV is of little if any threat to the blood supply at this point in time. Similar seroprevalence studies should be performed periodically to assess whether this will change in the future.

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