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The molecular epidemiology of hepatitis E virus genotype 3 in Canada

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Short Paper

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Abstract

Autochthonous hepatitis E virus (HEV) infection is increasingly reported in industrialized countries and is mostly associated with zoonotic HEV genotype 3 (HEV-3). In this study, we examined the molecular epidemiology of 63 human clinical HEV-3 isolates in Canada between 2014 and 2022. Fifty-five samples were IgM positive, 45 samples were IgG positive and 44 were IgM and IgG positive. The majority of the isolates belong to the subtypes 3a, 3b, and 3j, with high sequence homology to Canadian swine and pork isolates. There were a few isolates that clustered with subtypes 3c, 3e, 3f, 3h, and 3g, and an isolate from chronic infection with a rabbit strain (3ra). Previous studies have demonstrated that the isolates from pork products and swine from Canada belong to subtypes 3a and 3b, therefore, domestic swine HEV is likely responsible for the majority of clinical HEV cases in Canada and further support the hypothesis that swine serve as the main reservoirs for HEV-3 infections. Understanding the associated risk of zoonotic HEV infection requires the establishment of sustainable surveillance strategies at the interface between humans, animals, and the environment within a One-Health framework.

Introduction

Infection with hepatitis E virus (HEV) is responsible for acute hepatitis leading to an estimated 3.3 million new symptomatic cases and over 44,000 deaths annually around the world [1]. HEV, species *Paslahepevirus balayani*, belongs to the genus *Paslahepevirus* of the family *Hepeviridae* [2]. Eight genotypes have been defined, of which genotypes 1 and 2 only infect humans, and genotypes 3–6 infect mainly pigs and wild boars, with zoonotic spillover infections from genotypes 3 and 4 to humans and other mammals [3]. Genotypes 7 and 8 are mainly found in camelids [3]. Three clades are observed within HEV genotype 3 (HEV-3): group 1 (subtypes HEV-3e, f, and g), group 2 (HEV-3a, b, c, h, i, j, k, l, and m) and group 3, which includes rabbit (HEV-3ra) isolates. The frequency at which the various subtypes occur varies by region and can change over time ([4]).

The HEV genome is a single-stranded, positive sense RNA that encodes three open reading frames (ORFs): ORF 1 codes for the viral non-structural proteins including the RNA-dependent RNA polymerase (RdRP), ORF 2 codes for the major capsid protein, which is the main target for neutralizing antibodies, and the protein coded by ORF3 is believed to be involved in acquisition of lipid bilayers to form the quasi-envelope virion, allowing it to evade the immune system [5].

During the last two decades, HEV-3 has become an emerging cause of viral hepatitis in Western industrialized countries as an increasing number of autochthonous HEV infections have been recognized in many European countries [7]. Although HEV-3 has a wide host range, domesticated swine is considered to be the main reservoir and the most likely source of human infections [7].

Infection with HEV-3 causes subclinical or acute self-limiting hepatitis [8, 9]. However, chronic hepatitis is increasingly reported especially in immunocompromised patients, including solid organ transplant recipients, patients with malignancies and human immunodeficiency virus (HIV) infection [10, 11]. Furthermore, HEV infection can lead to extrahepatic complications, such as neurological complications, kidney injury, and haematological disorders [12–14].

The distribution of HEV subtypes in cohorts of patients with hepatic disease has been studied in a number of European countries such as the UK [15], Italy [16], Germany [17], the Netherlands [18], and Spain [19]. In Canada, HEV-3 infections in clinical cases and their molecular characteristics have not been described. However, several studies have reported the presence of HEV-3 genome in swine and cervid species as well as pork products [20–23].

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The seroprevalence of HEV among Canadian blood donors was 5.9% between 2013 and 2015 [24], which is comparable with the United States at 7.7–12.5% [25, 26] and considerably lower than many regions in Europe [27].

This study aims to characterize the clinical HEV-3 isolates submitted to the National Microbiology Laboratory (NML) from across Canada between 2014 and 2022 and investigate the possible sources of transmission by comparing the genetic relatedness of these isolates. For this purpose, we merged the sequences obtained from human, animals and food isolates to trace the origin of HEV-3 clinical strains circulating in Canada.

Materials and methods

Clinical sample description

Specimens are routinely submitted to the National Microbiology Laboratory (NML) of the Public Health Agency of Canada for serological and molecular testing of HEV as a standard of care. The NML is the reference diagnostic laboratory for Canada and provides HEV IgM/IgG serological testing, HEV RNA detection and quantification and HEV genotyping services to provincial public health laboratories (https://cnphi.canada.ca/gts/laboratory/1014). Serum or plasma samples are submitted based on suspicion of HEV infection. Only age and gender were available for the majority of reference requests, therefore travel and food history are also unknown.

HEV serological testing

Serum or plasma submitted to the NML for HEV serological analysis was tested using the Wantai HEV-IgM and HEV-IgG microplate enzyme immunoassays (Beijing Wantai BioPharm, Beijing, China) according to the manufacturer's instructions. Briefly, 10 µL specimen is added to microplates for solid-phase antibody capture by recombinant HEV protein (IgG assay) or anti-human IgM antibodies (IgM assay). Both assays involve a two-step incubation with enzyme-linked secondary antibodies allowing a chromogenic reaction to provide qualitative detection of antibodies. Sample reactivity is based on an assay absorbance to cutoff (A/CO) ≥ 1; non-reactive samples have an A/CO <1, and samples are considered borderline with A/CO between 0.9 and 1.5. Borderline specimens are re-tested in duplicate and tested for HEV RNA.

HEV RNA extraction and amplification

Viral RNA was extracted from 250 ul of serum/plasma using the Nuclisens EasyMag (Biomerieux) automated extraction system and eluted in 60 µL elution buffer. Extracted RNA was amplified using a previously described hemi-nested RT-PCR [28], targeting a 338 bp fragment of the RdRp region of HEV ORF1. Amplicons were purified using Amicon®Ultra filters (Millipore) and submitted for sequencing with the appropriate primers.

Pork isolates description

The Canadian pork isolates included in this study have been described previously [29]. Briefly, 15 HEV-3 isolates from pork paté from the 2014–2015 surveillance study referenced above were subjected to RNA extraction using TRI Reagent® and 1-bromo-3-choloropropane (Sigma-Aldrich), followed by RNA concentration using Dynabeads-Oligo (dT)25 (Thermo Fisher) following the manufacturer's instructions for purifying mRNA from total RNA. RNA was eluted in 25 µL of DNase/RNase-free water by heating the beads at 90°C for 2 min. The extracted RNA was subjected to RT-PCR using the primers and conditions described in [28].

HEV sequencing

HEV RT-PCR positive samples were further characterized by amplicon sequencing. The PCR products were gel purified using the QIAquick gel extraction kit (Qiagen) and sequenced in both directions with the nested PCR primers using the BigDye Terminator cycle sequencing kit (Applied Biosystems) and separated on a model 3730xl genetic analyzer (Applied Biosystems). Nucleotide sequences of PCR products were analyzed using BioEdit software (bioedit.software.informer.com). HEV sequences are available from https://www.ncbi.nlm.nih.gov/nucleotide/ at accession numbers OR978137–OR978214.

Phylogenetic analysis

Partial RdRp nucleotide sequences were aligned with select GenBank reference sequences representing HEV-3 subtypes from human and isolates from pork and swine [4] using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) [31] and trimmed to remove primer sequences. Maximum likelihood analysis of the ORF1 region was performed using MEGA X [32] with 1,000 bootstrap replications. Tree visualization and annotation were performed with MEGA X.

Results and discussion

Demographic profile of HEV-3 infected Canadians

From 2014 to 2022, the NML detected 63 unique cases of active HEV-3 infection (i.e., HEV RNA positive). Fifty-six of 63 specimens had associated serological data (Figure 1 and Table 1), with 94.6% (53/56) of the cases positive for HEV IgM. The majority of HEV-3 cases were male (71.2%; 42/59 having associated data) and the median and mean age, respectively, were 50 and 51 years (range 18–83 years). HEV-3 cases were observed among six provinces of

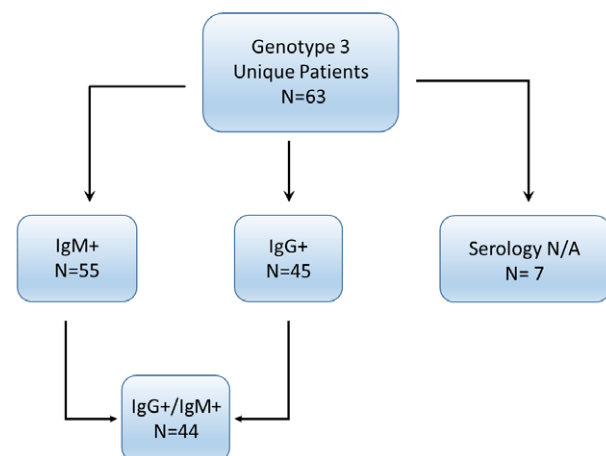

Figure 1. Serological data for the clinical samples, which were positive for HEV-3 RNA. '*n*' in brackets indicates the number of individuals.

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Table 1. HEV RNA positive study population by demographic and clinical characteristics, Canada 2014–2022

	Characteristics	*N*	%
Sex	Male	42	66.7
	Female	17	27.0
	Unknown	4	6.4
Province	Alberta	5	7.9
	British Columbia	7	11.1
	Manitoba	1	1.6
	New Brunswick	1	1.6
	Ontario	10	15.9
	Quebec	39	61.9
Serology	IgM+ IgG+	44	69.8
	IgM+ IgG−	9	14.3
	IgM− IgG+	1	1.6
	IgM−/IgG−	2	3.2
	N/A	7	11.1
Immunocompromised	Yes	11	17.5
	No	N/A	N/A
	Unknown	52	82.5

Canada between 2014 and 2022 (Table 1). Of note, of the three cases that were HEV IgM negative, further information was provided for two cases, indicating they were immunosuppressed.

Partial data based on RNA positivity for approximately 6 months indicated that 16 isolates belonged to chronic infection and the information regarding immunosuppression was available for 11 patients (Table 1). None of the replicate sequences were included in the phylogenetic analysis.

It is noteworthy that our observations regarding the sex (mostly male) and median age (≥50) in Canadian HEV-3 infected patients, are consistent with the trends observed in other studies from Europe and Japan [18, 19, 30].

Phylogenetic analysis

The phylogenetic tree constructed based on the partial ORF1 sequences from 62 HEV-3 (non-3ra) cases revealed that the majority of the clinical isolates are clustered with HEV-3 group 2 (subtypes a, b, j; Figure 1). There is 100% identity between two isolates from pork products (BMH-096D and BMH-047D) and two clinical isolates, H16-5242 and H15-2036 while there is 99.6% identity between those pork isolates and H18-1523, H16-2977, H15-4984, H15-1537, and H14-4574.

Another cluster of clinical sequences that show high similarity to pork isolates are H18-0125, H20-2424, H17-1749, H17-4401, H20-3946, and H19-5416, which are 96.25–97% identical to BMH-034.

Although H17-4115, H15-4981, H14-0583, and H20-2519 are not clustered with the pork isolates, they show high similarity to subtype '3j' reference AY115488, which is a swine isolate from Canada. The same is true for H14-0577, H14-2436, H20-2533, and H15-1508, which are clustered with KJ507956, a swine isolate

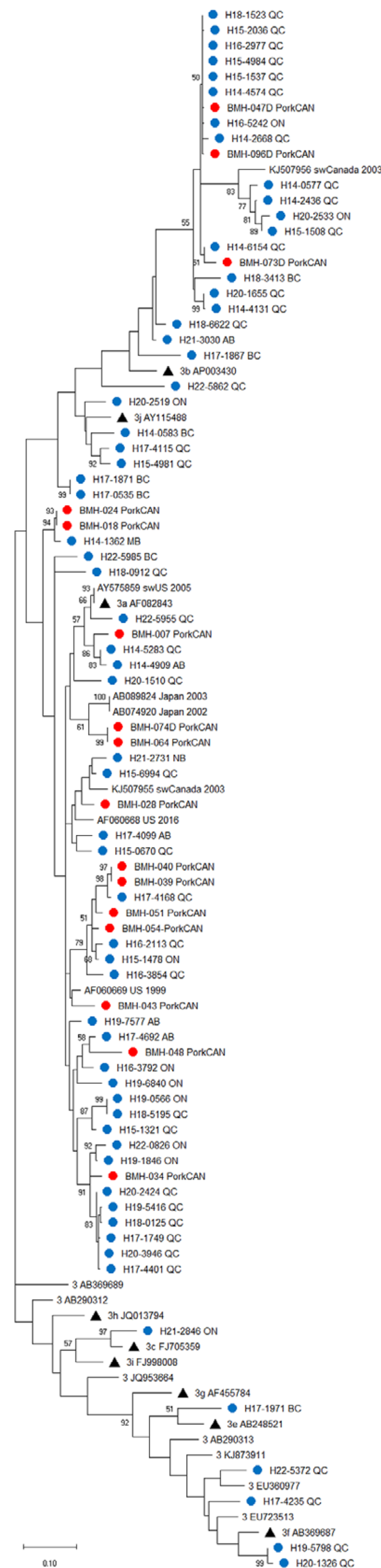

Figure 2. Phylogenetic analysis based on partial ORF1 region of HEV-3 strains of human or animal origin. The maximum likelihood tree was produced from the 338 bp fragment of the RdRp region, using 1,000 bootstrap replications. Bootstraps values >50 are indicated at their respective nodes The 63 clinical sequences from this study are indicated by blue circles and the pork isolates by red circles. The reference sequences are identified by black triangle. The accession numbers, country of origin and the year of isolation are also indicated.

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from Quebec. One isolate, H21-2846, is clustered with the subtype 3c, which is dominant in Europe [33] and could be associated with travel or the consumption of imported meat.

HEV-3 group 1 (subtypes e, f, and g) are mostly prevalent in Europe [33]. For example, the majority of infections in France belong to clades e, f, and g [34] and infections in Italy are mostly associated with subtypes 3f [16]. The Canadian pork isolates mostly belong to subtypes 3a and 3b [22, 23]; therefore, the presence of a few clinical isolates in Canada that belong to subtypes 3c, 3e, 3f, and 3g could be attributed to the consumption of imported meat products, or possibly travel to endemic regions of Europe. An extensive phylogenetic analysis of HEV-3 isolated from farmed pigs in Europe has also revealed that subtypes 3c, 3e, 3f, and 3g are most common, while there are only a few isolates that belong to 3a, and 3b [35]. Furthermore, it has been indicated that subtypes e, f, g are associated with a more severe disease presentation [29]. Interestingly, the majority of HEV-3 clinical cases reported in Japan belong to subtypes a and b [30]. Further epidemiological analyses are required to elucidate the similarity in HEV-3 isolates between distant regions.

Sporadic cases of human infections with rabbit HEV-3 (HEV-3ra) have been reported in Asia and Europe [36]. The majority of HEV-3ra infections in humans have occurred in immunosuppressed patients, demonstrating the zoonotic risk of HEV-3ra [36]. Herein, we also identified one HEV-3ra sequence isolated from a patients infected with H21-5187 QC (HEV IgM+ IgG+) who was later determined to have a chronic HEV infection as persistent HEV RNA was detected from 2021 to 2022 (Figure 2). There was no nucleotide change between the sequences obtained within this time span (data not shown). This isolate shows homology to sequences from France; however, the number of HEV-3ra sequences available from GenBank is limited and further molecular epidemiological studies are required for higher-resolution phylogenetic analysis. Several studies have shown the presence of HEV-3ra in commercial rabbits in Ontario (5% prevalence; [37]) and studied rabbits in Virginia, USA (49%; [38]). The targeted HEV genome region in those studies was not ORF1 and therefore could not be included in our phylogenetic analysis. However, these findings demonstrate that HEV-3ra is prevalent among rabbit populations in North America and could be the source of the clinical cases described here.

Consumption of certain pork products is currently considered a significant risk factor for developing HEV infection in developed countries [39] and our data also indicate that HEV-3 infections in Canada are highly associated with HEV present in pigs and contaminated pork products. The source of infection observed in this study may arise through occupational exposure to infected swine or the consumption of contaminated pork products. Therefore, this study provides an insight into the transmission route of autochthonous HEV infections in Canada. HEV seroprevalence has also been reported in cervids such as caribou, white-tailed deer and mule deer in Canada [21]; however, no RNA was detected, therefore no phylogenetic analysis could be performed.

In a zoonotic infection such as HEV-3, it is crucial to recognize the interconnections between humans, animals, and their shared environment and to implement a One-Health multisectoral approach to achieve optimal outcomes. It is estimated that globally, nearly 13% of domestic swine and 9.5% of wild boars are actively infected with HEV-3, and HEV-3 RNA is present in 5.5%–15.5% of global pork products [40]. At such high prevalence, the control measures against autochthonous HEV-3 infections require continuous collaborations among health authorities, veterinarians, clinicians and the food industry. For example, while several mitigation strategies such as treatments with organic acids and high-pressure processing are not completely effective against HEV in food products [41, 42], cooking to a minimum internal temperature of 72°C for at least 20 min is likely to inactivate HEV present in food matrices [39]. Additionally, improving sanitation and hygiene at pig farms and slaughterhouses has been linked to better outcomes in HEV-3 control [43].

A limitation of this study is the limited number of pork and swine isolates that could be included in the phylogenetic analysis. One reason for this is that the majority of pork and swine isolates in public sequence databases are specific to the ORF-2 region, which is not the region targeted in this study. Also, HEV is not a nationally reportable disease, although it is reportable at the provincial level in several provinces; thus, its prevalence is most likely under-reported, since Canadian physicians may not consider HEV as a possibility in their patients. Therefore, the number of clinical infections described in this study does not necessarily represent the full picture of HEV-3 incidence in Canada.

In summary, this study demonstrates the dominance of group 2 subtypes (3a, 3b, 3j) among HEV-3 strains observed in Canada, which are also reflected in the swine isolates (Figure 2). However,

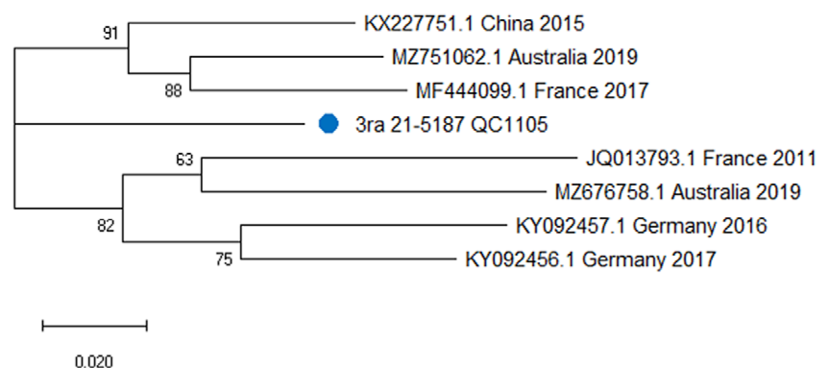

Figure 3. Phylogenetic analysis based on partial ORF1 region of HEV-3ra strains. The maximum likelihood tree was produced from the 338 bp fragment of the RdRp region, using the 1,000 bootstrap replications. The clinical sequence from this study is indicated by blue circle.

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sporadic infections with other genotypes such as 3c, 3f, 3g, 3ra have also been observed, while no isolate clustered with 3l or 3m subtypes. Future epidemiological studies with the inclusion of clinical, food, and environmental isolates could elucidate the potential role of HEV subtypes and strains on acute hepatitis, hospitalization, and chronic courses of HEV infections.

Overall, the results from this study provide a direct comparison of HEV-3 subtype distribution in humans and animals in Canada. Therefore, this work may pioneer a thorough exploration of the dynamics of HEV-3 transmissibility at the human-animal interface, based on a larger availability of shared HEV results in North America.

Data availability statement. The genomic data are all available at the NCBI repository as described in Materials and Methods under accession numbers OR978137–OR978214. Further epidemiological data of the de-identified cases could be requested from the authors.

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Author contribution. Conceptualization: C.O., N.N.; Data curation: C.O., J.B., J.H., N.N.; Funding acquisition: C.O., N.N.; Investigation: C.O., D.M., J.B., J.H., N.N.; Methodology: C.O., J.B., J.H.; Project administration: C.O., D.M.; Resources: C.O., D.M.; Supervision: C.O., N.N.; Writing – original draft: C.O., N.N.; Writing – review & editing: C.O., D.M.; Formal analysis: D.M., J.B., J.H.; Software: J.B., N.N.; Validation: J.B.; Visualization: J.B., N.N.

Ethical standard. The National Microbiology Laboratory (NML) is the national reference laboratory for diagnostic testing and surveillance of viral hepatitis in Canada. Specimens are routinely received for HEV serological and genotype testing as standard of care. Thus specimens received for diagnostic testing represent patients presenting with symptoms to their physician, presumed to be infected with HEV. Only age and gender were available for the majority of reference requests. All research was conducted according to the principles expressed in the Declaration of Helsinki.

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