Molecular epidemiology and clinical characteristics of hepatitis D virus infection in Canada

Carla Osiowy, Ken Swidinsky, Sarah Haylock-Jacobs, Matthew D. Sadler, Scott Fung, David Wong, Gerald Y. Minuk, Karen E. Doucette, Philip Wong, Edward Tam, Curtis Cooper, Alnoor Ramji, Mang Ma, Carmine Nudo, Keith Tsoi, Carla S. Coffin

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Supplementary materials and methods

HDV testing

HDV IgG antibody testing was performed using the Wantai HDV-IgG ELISA assay (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd, Beijing, China). The HDV IgG borderline interpretation was broadened to incorporate samples having optical density to cut-off ratios equal to 0.9 to 1.5. All borderline samples were repeated in duplicate to establish negative, positive or repeat borderline results.

To determine the presence of HDV RNA, 200 µL serum or plasma was extracted using an automated nucleic acid extraction system (NucliSENS easyMag, bioMerieux Inc., Saint-Laurent, QC) and eluted in 55 µL elution buffer. HDV RNA was amplified with outer primers HDV853f (5' GCCCAGGTCGGACCGCGAGGAGGT 3') and HDV1305r (5' ACAAGGAGAGGCAGGATCACCGAC 3') and inner, nested primers HDVf (5' ATGCCATGCCGACCCGAAGAGGAA 3') and HDVr (5' GAAGGAAGGCCCTCGAGAACAAGA 3'), adapted from Niro et al. [1997][1]. First stage outer amplification was performed using a one-step RT-PCR protocol (Qiagen Inc., Montreal, QC) with an initial reverse transcription cycle at 50°C for 30 minutes, followed by 35 cycles of amplification using an annealing temperature of 55°C, to result in a 466bp amplicon within the HDV antigen coding region (nt 856-1310 according to reference GenBank sequence M58629). The nested PCR reaction utilized *Taq* polymerase (Promega, Madison WI) and consisted of 35 cycles of amplification using an annealing temperature of 58°C to result in a 401bp amplicon. The risk of PCR environmental contamination was controlled with spatial and temporal separation of all steps (RNA extraction, PCR reaction pre-mix preparation, PCR amplification and nested PCR steps) as well as the inclusion of negative controls at the extraction and

amplification steps. The sensitivity of the nested PCR reaction was determined by triplicate testing of diluted preparations of the WHO HDV RNA standard (1st international standard, 2013; 7657/12) ranging from 3.76 $\log_{10}$ to 0.76 $\log_{10}$ IU/mL. The first stage reaction was reproducibly positive to a limit of 2.9 $\log_{10}$, while the nested reaction was consistently positive at 2.3 $\log_{10}$ IU/mL. There is no standard conversion between IU/mL and copies/mL for HDV RNA. A measure of 62 IU/mL equivalent to 1 copy/mL HDV RNA has been reported for a Cobas TaqMan based laboratory developed test[2]. A quantitative RT-PCR method for HDV viral load measurement, utilizing a synthetic RNA standard calibrated to the WHO IU/mL standard was used to determine the IU/mL conversion to copies/mL for the nested PCR limit of detection. The conversion was 1 copy/mL equivalent to 18 IU/mL, thus the nested PCR reaction reproducibly detected 11 copies/mL, with possible or intermittent detection to 3.2 copies/mL. Only HDV seropositive specimens were tested for HDV RNA.

Amplicons were purified and cycle sequenced using an Applied Biosystems 3730 XL DNA Analyzer (ThermoFisher Scientific, Burlington, ON). Sequences were aligned with GenBank reference sequences representing all HDV genotypes and subgenotypes using MAFFT[3] and trimmed. Maximum likelihood analysis of the partial delta antigen coding region (trimmed to approximately 357 bp representing nt 906 to 1263, based on GenBank accession M58629) was performed using DIVEIN software[4] by the most appropriate substitution model determined for the alignment by SMS Akaike Information Criterion[5]. Phylogenetic tree construction was optimized by nearest neighbour interchange and subtree pruning and regrafting with branch support computed by the approximate likelihood-ratio test based on a Shimodaira-Hasegawa-like procedure[6]. Clinical specimen HDV genotypes were ascertained by clustering with reference

sequences supported by branch support ≥70%. HDV subgenotypes were estimated by cluster location.

HBV genotype determination

HBV DNA from clinical specimens was extracted and amplified according to methods previously described[7, 8] for the determination of HBV genotype by line probe hybridization (INNO-LiPA HBV Genotyping, Fujirebio US Inc., Malvern, PA) or sequence analysis of the HBsAg coding region.

Quantitative HBsAg determination

Quantitative HBsAg testing for samples not tested at local public health laboratories was performed by the National Microbiology Laboratory using the Elecsys HBsAg II quant immunoassay (Roche Diagnostics Canada, Laval QC). The assay limit of detection is 0.05 IU/mL and the quantifiable range of the assay for undiluted samples is 0.05 to 130 IU/mL.

Ethical approval

Data from all patients enrolled in the Canadian HBV Network are received as anonymous and are collected under a protocol (Ethics ID# REB16-0041) approved by the University of Calgary Conjoint Health Research Ethics board (REB) and analyzed under the ethics protocol ID# REB17-2331. All clinic and hospital subsites have ethics and legal agreements for data sharing between sites with the Network. Eligible participants either provided consent, or were included with a waiver of consent, based on each site's local REB approval. The testing of Canadian HBV Network enrolled patient specimens at the NML was approved by the Health Canada and Public Health Agency of Canada REB (protocol ID REB 2019-036P) and the University of Manitoba

institutional ethics review board (protocol ID H2020:403). Linkage of anonymous data with HDV result data was assisted by the Network research coordinator.

Statistical analysis

ANOVA (1-way) was used to analyze the association of HDV seropositivity with age and Fisher's exact test (two-sided) was used to analyze the association of gender of tested individuals. Confidence intervals of prevalence estimates were calculated by computing the confidence interval of a proportion by the Wilson/Brown method. Continuous variable analysis among data derived from the Canadian HBV Network patient subset were summarized with the mean, 95% confidence interval (CI) and count (n), and comparisons between two groups were performed using t-tests or Mann-Whitney tests as explained in Table footnotes, or with ordinary one-way ANOVA tests where there were three comparison categories. Categorical variables were summarized using the proportion (%) and count (n/n known) and comparisons were performed using chi-squared test or Fisher's exact test as explained in Table footnotes. A p-value of less than 0.05 was deemed to be statistically significant. Statistical analysis was conducted using GraphPad Prism 9.0.0. Missing data was excluded from tables and figures.

Table S1: Comparison of age and gender for HDV seropositive and seronegative patients referred to specialists from 2012 to 2019.

	HDV seropositive (n= 338)	HDV seronegative (n= 6,736)	P value
Mean Age (95% CI, n)	44 (42.6-45.4, 337)	44.3 (43.9-44.6, 6722)	0.7165
Male sex[1]	69.3% (232/335)	61.7% (4105/6656)	0.1986

[1]3/338 seropositive and 80/6736 seronegative individuals had no gender designation. Significance of comparison among age was determined by Analysis of Variance testing, and among gender was determined by Fisher's exact test.

Table S2: List of countries with ≥5% prevalence of HBV were considered endemic[9]

Region	Countries with ≥ 5% prevalence of HBV.
WHO African Region	Angola, Benin, Burkina Faso, Burundi, Cameroon, Cape Verde, Central African Republic, Congo, Côte d'Ivorie, DR Congo, Equatorial Guinea, Ethiopia, Gabon, Gambia, Ghana, Guinea, Kenya, Liberia, Malawi, Mali, Mauritania, Mozambique, Namibia, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, South Africa, South Sudan, Swaziland, Togo, Uganda, Tanzania, Zambia, Zimbabwe
WHO Region of the Americas	Haiti
WHO Eastern Mediterranean Region	Djibouti, Oman, Somalia, Sudan, Tunisia, Yemen
WHO European Region	Albania, Kazakhstan, Kyrgyzstan, Moldova, Romania, Tajikistan, Uzbekistan
WHO South East Asian Region	Bhutan, Thailand
WHO Western Pacific Region	China, Kiribati, Laos, Marshall Islands, Mongolia, Nauru, Niue, Papua New Guinea, Samoa, Solomon Islands, Tonga, Tuvalu, Vanuatu, Vietnam

Table S3: Comparison of comorbidities in HBV mono-infected individuals (N=5,132) compared to HDV-HBV co-infected* individuals (N=135) followed by the Canadian HBV Network. *HDV-HBV = anti-HDV+ (and HDV RNA+ or HDV RNA-).

	HDV-HBV co-infected (n= 135)	HBV mono-infected (n= 5,132)	P value
Comorbidities			
Non-alcoholic fatty liver disease	10.4% (14/135)	6.4% (326/5132)	0.061
Diabetes	6.7% (9/135)	5.5% (284/5132)	0.571
Cardiovascular disease	1.5% (2/135)	1.4% (72/5132)	0.939
Hypertension	19.3% (26/135)	10.9% (561/5132)	0.002
Dyslipidemia	5.2% (7/135)	6.1% (312/5132)	0.667
Chronic kidney disease	3.7% (5/135)	1.4% (73/5132)	0.030
Osteoporosis	2.2% (3/135)	1.0% (50/5132)	0.152
Cancer (excl. HCC)	5.9% (8/135)	2.2% (112/5132)	0.004
Psychiatric	5.9% (8/135)	3.3% (167/5132)	0.087

T-test was used for continuous data, chi-square tests were used for categorical data. $P < 0.05$ considered significant. For both continuous and categorical variables missing data are excluded (n known is shown in table).

Table S4: Hepatic and virological outcomes and pegylated IFN-alpha treatment experience in HDV-HBV co-infected* individuals stratified by qHBsAg levels undetectable, <100 or >100 IU/mL (N=112). *HDV-HBV = anti-HDV+ (and HDV RNA+ or HDV RNA-).

	qHBsAg (IU/mL)			P value
	Undetectable	**< 100**	**> 100**	
n	15	13	84	
Mean IU/mL (95% CI)	N/A	27.12 (12-42.24)	ND[1]	
Median IU/mL	N/A	20.76	ND[1]	
Cirrhosis	53% (8/15)	46% (6/13)	43% (36/84)	0.749
Hepatocellular carcinoma	21% (3/14)	0% (0/12)	10% (8/80)	0.198
Transient Elastography (TE; kPa)	9.3 (4.4-14.2, 8)	12.8 (5.8-19.9, 13)	14.0 (10.9-17.2, 61)	0.559
HDV RNA negative	17% (2/12)	20% (2/10)	4% (3/75)	0.074
IFNa[2]	20% (3/15)	31% (4/13)	24% (20/84)	0.795
HDV genotype (n)				
1a	1	0	3	
1b	0	0	12	
1c	1	0	11	
1d	2	6	31	
2b	0	0	1	N/A
5a	0	0	9	
5b	0	0	2	
Unknown	2	1	2	
HDV RNA negative	9	6	13	

Continuous data are shown as mean (95% CI, n known). Categorical data are shown as mean % (n/n known). T-test was used, and chi-square tests were done for categorical data. $P < 0.05$ considered significant. [1]ND: not done, as values >130 IU/mL were not delineated. [2]IFNa treatment at any time.

Table S5: HDV genotype association with CanHepB cohort patient country of birth (n=117)

HDV genotype (n)	Birth country known n	Country of Birth
1a (5)	5	40% Eritrea, 40% Ethiopia, 20% Columbia
1b (13)	12	75% Sudan, 8.3% Kenya, 8.3% Cameroon, 8.3% Ethiopia
1c (12)	11	45.5% Mongolia, 18.2% Russia, 18.2% Afghanistan, 9.1% Pakistan, 9.1% Romania
1d (44)	41	*Asian* (n=12; 29.3%): 16.7% Korea, 16.7% Vietnam, 8.3% Pakistan, 58.3% Mongolia *White* (Canadian, n=12; 26.8%): 100% Canada; White (Other, n=15; 36.6%): 6.7% Georgia, 26.7% Moldova, 6.7% Portugal, 33.3% Romania, 13.3% Russia, 6.7% Switzerland, 6.7% Uzbekistan *Black/African/Caribbean* (n=1; 2.4%): 100% Canada *Other* (n=2; 4.9%): 100% Afghanistan
2b (1)	1	100% Russia
5a (12)	11	45.5% Liberia, 18.2% Sierra Leone, 18.2% Nigeria, 9.1% Ghana, 9.1% Guyana
5b (2)	2	50% Senegal, 50% Cote d'Ivoire
RNA Neg[1] (35)	29	*Asian* (n=7; 24.1%): 28.6% Mongolia, 14.3% China, 14.3% Taiwan, 14.3% Korea, 14.3% Pakistan, 14.3% Philippines *White* (Canadian, n=2; 6.9%): 100% Canada; White (Other, n=3; 10.3%): 66.7% Romania, 33.3% Ukraine *Black/African/Caribbean* (n=15; 51.7%): 26.7% Sudan, 20% Liberia, 13.3 % Nigeria, 13.3% Ethiopia, 6.7% Cameroon, 6.7% Congo, 6.7% Ghana, 6.7% Somalia *Other* (n=2; 6.9%): 50% Columbia, 50% Iraq
RNA Pos[1] no seq[1] (6)	5	20% Korea, 20% Afghanistan, 20% Kazakhstan, 20% Romania, 20% Canada

[1]Neg; negative, Pos; positive, seq; sequence.

Table S6: HDV genotype (gt) associations with severe liver outcomes.

	1a (n=5)	1b (n=13)	1c (n=12)	1d (n=44)	2b (n=1)	5a (n=12)	5b (n=2)	gt unknown[1] (n=46)	Total
HCC	1	1	0	4	0	2	0	3	11
Cirrhosis	3	7	6	26	0	4	1	14	61
Liver transplant	1	1	0	1	0	1	0	5	9
Decompensation[2]	3	2	0	8	0	3	1	4	21
Any combination[3]	3	7	6	27	0	4	1	16	64

[1]Includes 35 RNA negative, 5 RNA unknown, and 6 RNA positive non-genotyped participants. [2]Indicators included were jaundice, ascites, variceal bleeding, hepatic encephalopathy. [3]Includes HCC, cirrhosis, liver transplant, and/or decompensation.

Table S7: HDV-HBV genotype associations among co-infected patients.

		HBV genotype (n)						
		A (8)	B (3)	C (3)	D (29)	E (11)	DNA Neg[1] (57)	NSQ[2] (19)
HDV genotype (n)	1a (5)				3		2	
	1b (13)				2	2	6	3
	1c (12)	1			2		8	1
	1d (44)	4	1		15		19	5
	2b (1)						1	
	5a (12)					5	5	2
	5b (2)					1	1	
	RNA Positive Genotype unknown (6)				2			4
	RNA Negative (35)	3	2	3	5	3	15	4

[1]Neg; negative. [2]NSQ; non-sufficient quantity to test.

Supplementary references

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