Journal of Clinical Virology 141 (2021) 104870

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Prevalence of hepatitis delta virus among chronic hepatitis B carriers in a large tertiary center in the Netherlands

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ARTICLE INFO

Keywords:
Hepatitis B
Hepatitis delta
Prevalence
Epidemiology
Screening
Genotyping

ABSTRACT

Background & aims: Hepatitis D virus infection (HDV) is considered the most severe form of viral hepatitis. In this study, we aimed to evaluate the prevalence of HDV infection in a tertiary center of a large, multi-ethnic city in the Netherlands. Moreover, we validate the reliability of a novel anti-HDV CLIA assay.

Methods: All HBsAg-positive patients visiting the outpatient clinic between 2017 and 2019 were tested for HDV serology. Seropositive serum samples were further assessed by HDV RNA PCR and Sanger sequencing to identify the HDV genotype.

Results: The CLIA assay was 100% sensitive and 98% specific. Out of 925 patients 3.7% tested seropositive for HDV, and HDV viremia was confirmed in 2.0%. The majority of patients had a non-Dutch background and did not speak English or Dutch. We detected HDV genotype 5 ($N = 3$), and genotype 1 ($N = 15$). Phylogenetic analysis demonstrated HDV1 clusters composed of sub-Saharan Africa isolates, central Asian, Turkish, Iranian and European isolates.

Conclusions: The prevalence of HDV infection in a tertiary center in the Netherlands was 2.0% among HBsAg-positive individuals, and mainly in non-Dutch individuals. Only HDV genotype 1 and 5 isolates were detected, which was found to match with the patient's country of origin.

1. Introduction

Hepatitis delta virus (HDV) infection is an important cause of liver-related morbidity and mortality in patients infected with hepatitis B (HBV). There is no consensus on global HDV prevalence, however recent estimates indicate that up to 12 to 50 million individuals are chronically infected with HDV worldwide [1,2]. Important risk factors for HDV infection are intravenous drug abuse (IVDU), men having sex with men (MSM), sex work, and hemodialysis treatments. HDV infection only occurs in HBsAg-positive individuals as the viral life cycle of this satellite RNA virus requires hepatitis B surface antigen (HBsAg) as an envelope protein to mediate viral entry into hepatocytes [3]. Transmission occurs either as coinfection after simultaneous transmission of HBV and HDV, or as superinfection in patients with pre-existing HBV infection. Chronic co-infection with both viruses is considered to lead to the most severe form of viral hepatitis, because of rapid progression to cirrhosis, and higher rates of hepatocellular carcinoma and liver-related mortality compared to that of HBV mono-infection [1,4,5]. In order to prevent HDV infection, there is a dire need for implementation of surveillance strategies in the patient population at risk. Currently, novel HDV treatments like entry inhibitor, Bulevirtide and the prenylation inhibitor, Lonafarnib are currently in phase III trials [6], and potentially improve the prognosis of these patients, emphasizing the relevance of HDV screening.

The prevalence of HDV infection is generally higher in low-income countries and regions where HBV is endemic. Estimates of anti-HDV rates among HBsAg-positive people are as high as 60% in Mongolia, and 10–30% in Western and Central Africa, the Amazon basin and Eastern Europe [1]. Prevalence studies struggle with the limited available data on HDV testing. Moreover, comparability and standardization of HDV RNA and anti-HDV assays is a real concern [1,7,8], which warrants the need for validation of in-house HDV RNA PCR and anti-HDV assays.

In this study, we determine the prevalence of HDV infection among

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https://doi.org/10.1016/j.jcv.2021.104870
Received 9 December 2020; Received in revised form 5 May 2021; Accepted 11 May 2021
Available online 18 June 2021

HBV patients in a large tertiary center in the Netherlands and validate the diagnostic performance of the novel LIAISON® XL murex anti-HDV assay (CLIA).

2. Material and methods

2.1. Study participants and samples

Serum and EDTA plasma were obtained from all chronic HBsAg-positive adults who visited the outpatient clinic of the Erasmus MC University Medical Center, Rotterdam, and the Netherlands, for routine clinical and virological assessment. Upon the first visit between January 1, 2017 and January 31, 2019 a sample was collected and stored in our −20 °C biobank; and we registered the clinical and biochemical characteristics of viremic (HDV RNA positive) patients.

Screening patients

In order to minimize referral and selection bias anti-HDV antibodies were tested in all unique HBV patients who visited the outpatient clinic. As part of standard regular care, HBV patients have follow-up visits at the outpatient clinic every 6–12 months, regardless of having symptoms. Patients, whom diagnostic evaluation between 2017 and 2019 did not include an anti-HDV test, were tested in the CLIA. RT-PCR for HDV RNA was conducted to confirm HDV viremia. All patient samples and data used in this study were collected in the context of routine clinical patient care and the ethical review board of our institution approved of the use of these data and samples (METC-2015–306).

2.3. Validation of the LIAISON® XL anti-HDV assay

The clinical sensitivity was evaluated of the chemiluminescent LIAISON® XL murex anti-HDV assay (CLIA) produced by DiaSorin S.p.A. in Italy (Lot#: 257005; REF#: 311260) [9]. It is a fully automated, indirect chemiluminescent immunoassay (CLIA) and it replaces our reference method, the ETI-AB-DELTAK-2 (EIA) anti-HDV assay (DiaSorin S.p.A., Italy; Lot#: 8690710A; REF#: P2808). The CLIA detects total anti-HDV in the range of 0.1 to 30.0 AU/mL. Serum or EDTA plasma samples with results above or equal to 1.0 AU/ mL are considered positive, according the manufacturer. Sensitivity was assessed by measuring antibodies to HDV in 21 HDV RNA carriers with various HDV genotypes. For diagnostic specificity testing, a panel of serum/ EDTA plasma of patients with common acute hepatitis viruses was selected: acute HBV ($n = 20$); acute hepatitis A virus ($n = 4$); acute Parvo B19 virus ($n = 5$); acute hepatitis E virus ($n = 5$), acute Epstein–Barr virus ($n = 5$) and acute cytomegalovirus ($n = 5$) infected patients. Samples from individuals who had either cleared HBV ($n = 5$) or from healthy individuals vaccinated for HBV ($n = 5$) were included. In addition, we repeated the samples of patients that tested HDV RNA positive or anti-HDV positive in the EIA in 2017–2019.

2.4. HDV real-time PCR

Total nucleic acids were extracted from 200 µl sample spiked with a known concentration of Phocine Distemper Virus (PDV, REF PMID 22,992,129) as internal control using the MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche) and the HP200 extraction protocol. Nucleic acids were resuspended in a final volume of 100 µl. Subsequently, 8 µl eluate was amplified in a 20 µl reaction containing 5 µl 4x Fast Viral Master Mix with UDG (FVMM, Thermo Fisher Scientific), 18 pmol HDV fwd primer (GAGGAGGTGGAGATGCCATG), 14 pmol HDV rev primer (CGGGTTTCCACTCACAGGTT), and 2 pmol HDV probe (6FAM_TCGCGTCCTTCTTTCCTCTTCGGGT-BHQ1). The reaction conditions were 5 min 50 °C, 20 s 95 °C, 45 cycles of 3 s 95 °C and 30 s 60 °C as thermal profile. Amplification was performed using a LightCycler 480 II (Roche) using the Fit points analysis module. Validation of the procedure was performed according to ISO 15,149:2012 guidelines. Negative RT-PCR results from serological positive patients were confirmed with a RealStar HDV RT-PCR Kit 1.0 (Altona), performed as described by the manufacturer.

2.5. Sanger sequencing

All HDV RNA positive samples were considered for genotyping if Ct value HDV RT-PCR <30, if Ct >30 a sample earlier in infection was used. For sequencing 8 µl eluate was amplified using PrimeScript One Step RT-PCR Kit ver.2 (Takara Bio), 8 pmol HDV_seq_fwd2 (CGAGTGAGGCTTATCCCGG), and 8 pmol HDV_seq_rev2 (GAAGGCCCTCGAGAACAAGA). The reaction conditions were 30 min 50 °C, 2 min 94 °C, 40 cycles of 30 s 95 °C, 30 s 55 °C, 2 min 72 °C and 5 min 72 °C as thermal profile. If necessary nested PCR was performed to increase sensitivity. In this case, 2 µl of the initial PCR was amplified using Hotstar High Fidelity Kit (Qiagen), 20 pmol HDV_seq_fwd (GCATGGTCCCAGCCTCC), 20 pmol HDV_seq_rev2 primer. The thermal profile was 5 min 95 °C, 30 cycles of 15 s 95 °C, 1 min 50 °C, 2 min 72 °C and 10 min 72 °C as thermal profile. Positive products were sequenced using the BigDyeTM Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) and a 3130xl genetic analyser (Applied Biosystems). Sequences were analyzed using SeqMan Pro-lasergene 10 software (DNA star) and genotypes were determined by phylogenetic analysis using MEGA_X, using the Maximum-likelyhood method and Kimura-2 parameter.

2.6. Statistical analysis

Continuous variables are expressed as mean with standard deviation or median with interquartile range (IQR). We tested associations between variables using Student's *t*-test, Chi-square, Pearson correlation or their non-parametric equivalents when appropriate. Sensitivity, specificity, disease prevalence, positive and negative predictive value are expressed as percentages. Confidence intervals for sensitivity, specificity are "exact" Clopper-Pearson confidence intervals.

3. Results

3.1. Epidemiology of HDV infection in a tertiary center of a large, multi-ethnic city in the Netherland

Between 2017 and 2019, 925 unique HBsAg positive patients visited the Erasmus MC. As part of regular diagnostic care, 636 patients were screened for HDV antibodies with the EIA. The remaining 283 patients were evaluated for HDV antibodies using the CLIA. Out of 925 patients, serum samples of six patients were not available in the biobank and were excluded from further analysis. Thirty-four patients (3.7%) tested seropositive for HDV, and we confirmed HDV viremia in 18 patients (2.0%). The characteristics of the viremic patients are shown in Table 1. The vast majority (94%) of patients had a non-Dutch background; they were predominantly from European (59%), African (24%), and Middle-eastern (18%) descent, but not with an Asian or South-American background. The majority of European patients had migrated to the Netherlands from Eastern Europe (80%) and were either Bulgarian, Turkish, Moldavian or Romanian nationals. Among the HDV carriers, 76% were unable to speak Dutch or English. Three patients were IVDU, of whom two were coinfected with hepatitis C and one with both hepatitis C and HIV. None of the other patients had documented risk factors like MSM, sex work, surgical treatments in foreign countries, blood transfusions or hemodialysis treatments. Two had hepatocellular carcinoma Barcelona Clinic Liver Cancer stage C, and 94% of the patients were on nucleoside analogue therapy for their HBV infection. Eleven patients had Metavir score four (liver cirrhosis), while one patient had F3 severe fibrosis, three patients had F2 moderate fibrosis, and three patients had F0-F1, no or minimal fibrosis.

Table 1
Patient characteristics of the 18 HDV RNA positive patients. Arranged according phylogenetic proximity, as depicted in Fig. 1A.

Patient	Name in phylogenic tree	HDV Genotype	Ethnicity	Age (years)	Sex	Documented IVDU, MSM, Sex work, Dialysis	Other Co-infections	ALT (U/L)	Total Bilirubin (μmol/L)	HBV DNA (IU/mL)	Metavir score
1	EMC 03	HDV-1	European	54	Male	IVDU	HCV	16	9	undetectable	F4
2	EMC 18	HDV-1	European	41	Male	None	None	112	12	undetectable	F3
3	EMC 07	HDV-1	European	38	Male	None	None	200	15	undetectable	F2
4	EMC 15	HDV-1	European	44	Male	IVDU	HIV, HCV	12	70	undetectable	F4
5	EMC 10	HDV-1	European	63	Male	None	None	31	10	undetectable	F4
6	EMC 17	HDV-1	European	37	Female	None	None	39	11	undetectable	F2
7	EMC 09	HDV-1	European	40	Male	None	None	143	9	undetectable	F4
8	EMC 14	HDV-1	European	32	Male	None	None	68	15	undetectable	F4
9	EMC 06	HDV-1	European	49	Female	None	None	174	4	undetectable	F2
10	EMC 08	HDV-1	Middle-Eastern	53	Male	None	None	183	14	undetectable	F4
11	EMC 05	HDV-1	Middle-Eastern	61	Male	None	None	24	6	undetectable	F4
12	EMC 11	HDV-1	Middle-Eastern	39	Female	None	None	54	24	undetectable	F4
13	EMC 12	HDV-1	Sub-Saharan Africa	57	Female	None	None	39	9	2.3	F4
14	EMC 16	HDV-1	Sub-Saharan Africa	56	Male	None	HIV	76	20	50,500	F0-F1
15	EMC 02	HDV-5	Sub-Saharan Africa	40	Male	None	None	31	12	undetectable	F0-F1
16	EMC 01	HDV-5	Sub-Saharan Africa	35	Male	None	None	148	24	undetectable	F4
17	EMC 04	HDV-5	Sub-Saharan Africa	36	Male	None	None	99	10	undetectable	F4
18	EMC 13	Undetermined	European	28	Female	None	None	59	12	undetectable	F0-F1

Abbreviations: HDV; hepatitis delta virus, IVDU; intravenous drug use, MSM; men having sex with men, ALT; alanine transferase, HBV DNA; hepatitis B virus Deoxyribonucleic acid.

3.2. Validation of anti-HDV CLIA assay

In order to assess the sensitivity and specificity of the CLIA, a panel of viremic HDV patients and a cross-reactivity panel was tested. The CLIA was 100% sensitive (95% CI: 83% to 100%) in viremic HDV patients. The CLIA was 98% specific (95% CI: 89% - 99%) as we obtained one false positive result in a patient with an acute HBV infection in 2017. We confirmed this result on two HDV RNA negative samples from the same patient, collected earlier in 2008 and 2018. The same samples tested negative on the EIA.

Next, we wanted to validate the performance of the CLIA in HDV RNA positive and negative samples identified through our screening effort. Seventeen HDV RNA PCR positive patients had positive anti-HDV serology in the CLIA (median CLIA level 8.7 AU/mL (8.5–8.9 AU/mL)). We identified one seropositive patient (CLIA level 8.3 AU/mL) who had been treated with peginterferon-α, resulting in undetectable HDV RNA serum levels. We considered this patient HDV positive. Eight EIA anti-HDV positive, HDV RNA negative samples were tested twice in the CLIA; these samples were repeatedly CLIA negative, and were therefore considered as EIA false positive results. Eight CLIA anti-HDV positive, HDV RNA negative samples were repeated e in the CLIA. Five of these gave concordant, repeatedly positive CLIA results (median CLIA level 1.47 AU/ml, range 1.15–5.49), and their anti-HDV status was confirmed by CLIA testing of samples taken years before screening; three samples were repeatedly CLIA negative, and were therefore considered as EIA false positive results. The final 3 samples were initially low level CLIA positive, but on repeat testing gave a median level of 0.42 AU/ml, range 0.35–0.43), suggesting the initial CLIA results were false positive

3.3. HDV real-time RT-PCR

It has been reported that several laboratories underestimated and/or failed to detect several HDV genotypes (i.e., HDV-5 to −8 and HDV-1 Afr [8]). Our alignment of primer/probe sequences against whole-genomic sequences of all HDV genotypes showed primer/probe mismatches; therefore, RT-PCR negative and serological positive samples were confirmed using the RealStar® HDV RT-PCR Kit 1.0. No extra positive samples were found.

We were able to genotype 18 RT-PCR positive patients, 17 by phylogenetic analysis (Fig. 1A). One sequence (EMC_13) was too short and hasn't been included into the polygenetic analysis.

Fourteen patients were infected with HDV genotype 1, which is the dominant genotype in Europe, and 3 patients were infected with genotype 5, which is mainly found in individuals from African origin. Recently it has been reported that there is a possible existence of specific geographic clusters within HDV genotype 1 [10]. Of our 15 HDV1 positive patients, 3 clustered within the sub-Saharan Africa cluster (HDV1a and 1b), 4 in the Central Asia cluster (HDV1c) 1 in the Turkey and Iran cluster (HDV1d) and 5 in the European cluster (HDV1e) [10, 11]. One patient (EMC 05, HDV1) didn't cluster with any of the geographic regions.

4. Discussion

Data on HDV prevalence and the subsequent risk of advanced liver disease among general populations and specific population groups are critical to guide clinical care and prevention strategies. In this study, we evaluated the prevalence of HDV infection in a tertiary center of a large, multi-ethnic city in the Netherlands, and we validated the reliability of a recently developed anti-HDV CLIA assay.

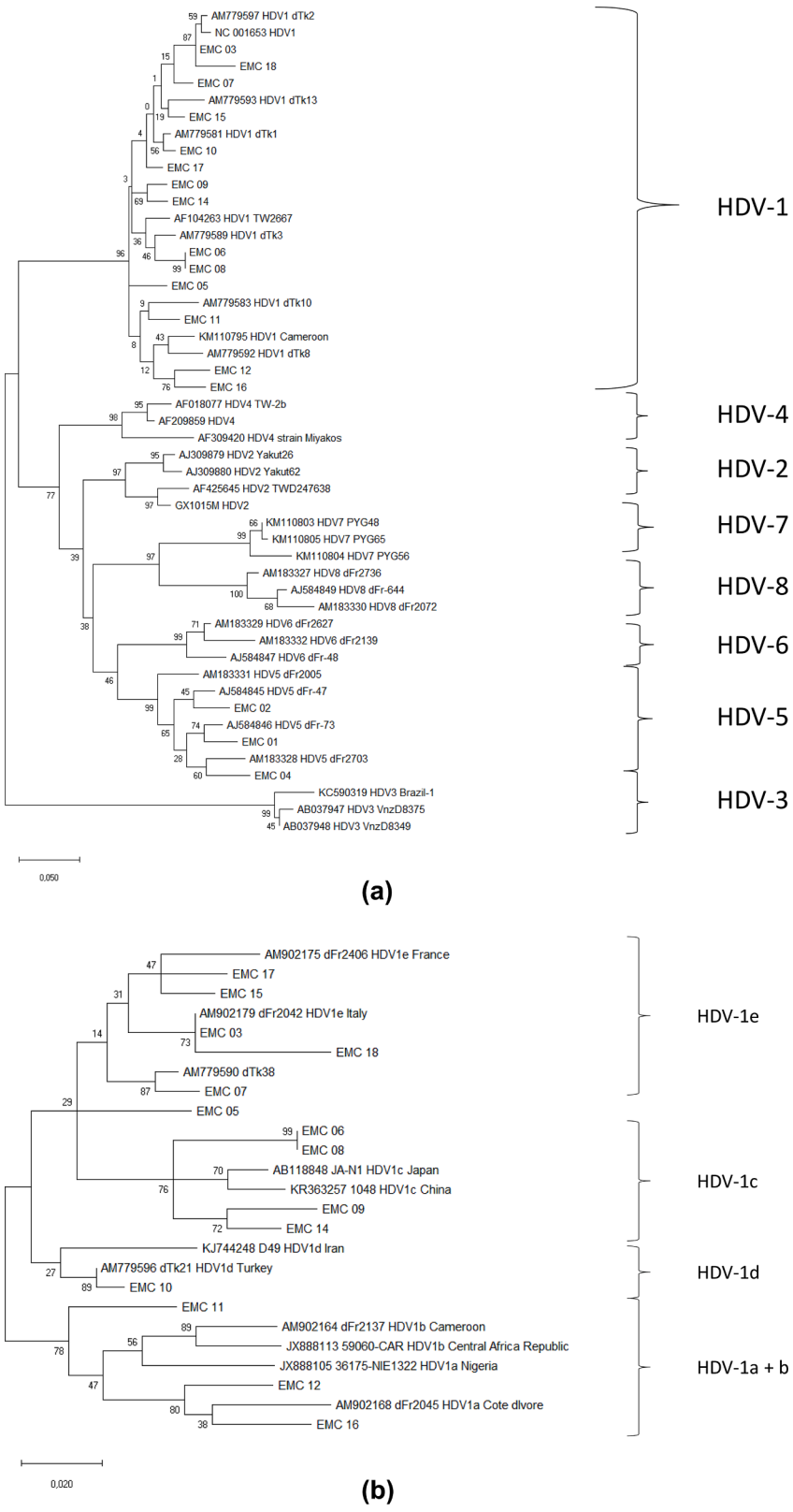

Fig. 1. A. Phylogenetic tree of 17 viremic Erasmus Medical Center (EMC) patients. The horizontal branch was drawn in accord with the relative genetic distance. A number of commonly used reference delta-gene sequences, for classifying eight HDV genotypes, were also included and are indicated by accession numbers. B. Phylogenetic tree with specific geographic clusters within HDV genotype 1. The horizontal branch was drawn in accord with the relative genetic distance. A number of commonly used reference delta 1-gene sequences were also included and are indicated by accession numbers.

Our findings show that the HDV prevalence is 2.0% of the HBsAg-positive patients visiting the Erasmus MC in Rotterdam, The Netherlands by studying serum of patients collected between 2017 and 2019. We believe this finding is robust and representative for the Rotterdam population, because as part of regular care, HBV patients have follow-up visits at the outpatient clinic every 6–12 months, regardless of having symptoms. Moreover, additional HDV testing was performed on HBV patients who had not been tested for HDV in the period between 2017 and 2019, thereby reducing selection bias and referral bias.

During the past decades, many immigrants from developing countries have settled in Europe with a surge in migration after the 2015 refugee crisis [12], which necessitates the need to identify putative

increases in the prevalence of transmittable infectious diseases due to this increased migration. This is the first study that reports HDV prevalence in the Netherlands. Our results are comparable to that of a French study that ran in 1997–2011, which found an anti-HDV prevalence of 2% among French blood donors who tested HBV positive. They subsequently detected circulating HDV RNA in 69% of the anti-HDV positive patients [13]. European studies have been reported on HDV prevalence dating back to cohorts in 2011 [13] and 2012 [14], however they predate the 2015 refugee crisis [12]. In Rotterdam, a city with about 650, 000 inhabitants, up to 50% has a non-Dutch ethnicity, including populations from Asia, South-America, Eastern Europe and Africa [15]. We found that HDV infection was mainly detected in patients who had migrated to the Netherlands from Eastern Europe, West Africa, Central Africa or the Middle East. The majority of them (76%) experienced a language barrier. It is unclear whether the language barrier affected access to healthcare, and whether this influenced disease progression. However, the majority of patients had advanced liver fibrosis. Only few patients had documented risk factors, like IVDU, but not MSM, sex work or hemodialysis treatments. Importantly, our findings do not demonstrate higher HDV rates in specific Dutch or non-Dutch populations.

Phylogenetic analysis of 17 HDV sequences indicated that all strains detected belonged to genotype HDV1 or HDV5, including difficult to detect strains HDV-5 and HDV-1 Afr [8]. The HDV isolates clustered with sequences from Western, Eastern Europe and the Middle East or sub-Saharan Africa [1,10,11] and corresponded with the geographic origin of the patient, suggesting that patients acquired their HDV infection in their country of origin.

The validation of the CLIA demonstrated that it was 100% sensitive. The specificity -tested with a cross-reactivity panel- was 98%. Importantly, the HDV RNA negative samples with a positive serological response showed median anti-HDV levels lower than in viremic patients. It is known that up to 50% of anti-HDV positive patients may have undetectable HDV RNA [4,13]. When using specific antibody tests and validated pan-genomic HDV RNA PCR assays, persistently higher anti-HDV levels may indicate a cleared HDV infection or sustained response as anti-HDV IgG may persist long-term, even after viral clearance. Because we have not been able to prove viral clearance in HDV RNA negative samples, a false positive result cannot be excluded. Adjusting the detection limit of the CLIA from 1.0 AU/mL to 2.0 AU/mL would mean losing six out of eight HDV RNA negative patients. Future research with HBV patients who cleared their HDV infection will have to validate this finding.

In conclusion, the prevalence of HDV infection in a tertiary center of a large, multi-ethnic city in the Netherlands was 2.0% and mainly associated with non-Dutch individuals. Only HDV genotype 1 and 5 isolates were detected, which was found to match with the patient's country of origin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank our experienced technician (Tim Rugenbrink, Department of Virology, and Erasmus MC University Medical Center) for the assistance with the molecular analysis. This study was supported by the European-Latin American ESCALON consortium, funded by the EU Horizon 2020 program, project number 825510 and was sponsored by the Foundation for Liver and Gastrointestinal Research (SLO).

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