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The current challenges underlying hepatitis D virus infection

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The current challenges underlying hepatitis D virus infection

Topic editors

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Editorial: The current challenges underlying hepatitis D virus infection

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HDV, HBV, liver cirrhosis, hepatocellular carcinoma, Bulevirtide

Editorial on the Research Topic
The current challenges underlying hepatitis D virus infection

The Hepatitis Delta virus (HDV) is the smallest human virus, causing the most severe viral hepatitis, in association with its co-helper virus, the Hepatitis B virus (HBV), on which it depends for the release of its progeny and *de-novo* entry into hepatocytes (1). Chronic HDV infection (CHD) is associated with a faster progression to cirrhosis and hepatocellular carcinoma, resulting in an increased fatality rate (2, 3). Despite its clinical relevance, CHD has been largely under-investigated for many years with relevant knowledge gaps concerning its epidemiology and pathogenic mechanisms.

This scarcity of data is explained by the limited therapeutic options available for several years. Until 2020, the only available therapeutic agent for HDV was interferon-alfa, a drug associated with a poor virological response and a high rate of post-treatment virological relapse (4). More recently, the approval of the entry inhibitor, Bulevirtide, together with the ongoing development of novel promising antiviral strategies, has led to a renewed focus around HDV and has prompted several studies aimed at better defining HDV epidemiology (5, 6). In this regard, it is relevant that the global prevalence of CHD still represents a subject of debate, with recent metanalyses reporting between 12 and 70 million HDV-infected subjects worldwide (7, 8).

Furthermore, it is noteworthy that factors underlying HDV pathogenesis are largely unknown. Particularly, the contribution of HDV-related immunological dysfunction and HDV genetic variability in modulating HDV disease progression remain poorly understood (9, 10).

Thus, this Research Topic was designed to provide new insights into HDV epidemiology and its molecular features, particularly in countries with limited data and in special high-risk populations. Moreover, the current research also examines mechanisms underlying HDV pathogenetic potential, focusing on virological and immunological factors. Overall, it comprises 7 original research articles and 3 brief research reports.

On a global perspective, there is evidence of wide heterogeneity in HDV prevalence across different areas of the world, with specific high-endemicity hospots. Among them, Mongolia is recognized as the country with the highest national anti-HDV prevalence among HBV-infected patients (ranging from 35% in the general population to 83% in high-risk groups). However, there is a paucity of studies on HDV molecular epidemiology in Mongolia. In this light, by investigating a large set of HDV sequences, Magvan et al.

showed that HDV isolates from Mongolia are characterized by a remarkably high genetic variability, with multiple HDV subgenotypes-1 belonging to different clusters and still unclassified subgenotypes. Moreover, this study has revealed that one of the driving forces of HDV genetic diversity is represented by viral adptation to HLA-class-I selective pressure. This concept poses the basis for the potential selection of viral-escape variants that could challenge the success of T-cell immunotherapies under development, this will need careful consideration in future clinical trial design.

Significant data gaps on HDV seroprevalence characterize Latin America, challenging the accurate estimates of HDV circulation in this region. In central Argentina, the study by Castro et al. showed for the first time a relevant rate of HDV seropositivity (5.2%) among HBsAg carriers, with the co-circulation of HDV-genotype 1 and HBV-genotype D3, highlighting the need to promote HDV screening campaigns.

Conversely, the study led in Cuba by de los Ángeles Rodríguez Lay et al. demonstrated a completely different epidemiological scenario in this country, with a limited HDV seroprevalence (0.4%), representing a major decline respect to the 8% reported in 1988. This notable decrease can be explained by the success of the Cuban anti-HBV vaccination program introduced in 1992, confirming that, as in other countries, universal anti-HBV vaccination represents an effective strategy to constrain HBV circulation and to limit HDV spread.

Two studies, respectively from USA (Argirion et al.) and Italy (Nicolini et al.), provided new data in favor of a large HDV circulation among HBsAg+ people living with HIV (PLWH) (22% and 15% respectively), thus confirming that PLWH represents a high-risk group for HDV infection. Moreover, Nicolini et al. showed that there is a not negligible proportion of HBsAg+PLWH remaining untested for anti-HDV, thus lacking HDV diagnosis. This percentage is even higher among non-HIV HBsAg+ patients from Southern Italy (35% in Fasano et al.) reflecting suboptimal HDV diagnosis. In this light, implementing HDV screening campaigns is pivotal, especially in light of the new anti-HDV drugs on the horizon.

This issue is particularly critical considering data from the European PLWH cohorts (Swiss HIV Cohort Study, the EuroSIDA Study and the French HIV/HBV cohort), published in this Research Topic by Begrè et al., demonstrating that HDV coinfection represents the leading independent risk factor for persistent ALT elevation during long-term tenofovir treatment in PLWH. This evidence further reinforces the need to carefully monitor liver disease progression in this high-risk setting.

This Research Topic also published novel concepts on an area poorly explored: HDV-related immunopathogenesis. In particular, Joshi et al. demonstrated an increased inflammatory response characterizing patients with CHD and a weak HBV and HDV specific T-cell response, confirming the HDV role in driving a state of "immune activation," contributing to liver damage.

Furthermore, the study from Hermanussen et al. provided new evidence on immune-mediated liver damage showing that, besides stimulating a state of immune-activation, HDV can also induce auto-immunity phenomena, that could also mediate extra-hepatic inflammatory manifestations.

Lastly, Hercun et al., by analyzing 50 liver biopsies from CHD patients, revealed for the first time the existence of a peculiar membranous HBsAg staining pattern characterizing patients on anti-HBV nucleos(t)ide analogs, that could potentially represent an indirect HBsAg diversion toward HDV replication. However, further understanding of this phenomenon, in addition to the role exerted by anti-HBV treatment in HDV co-infection is mandated to improve our understanding of HDV pathophysiology.

Overall, these articles depict a variegated and evolving epidemiological scenario for HDV infection, underlining the need to expand HDV screening programs worlwide, in order to increase diagnoses and improve the management of patients with CHD. Furthermore, the published studies on HDV pathogenesis highlight the importance of improved knowledge of the mechanisms underlying HDV-mediated immune activation since these critical insights could provide key information in the development and optimization of novel immune-based anti-HDV therapies.

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Long-term trends of alanine aminotransferase levels among persons living with human immunodeficiency virus/hepatitis B virus with and without hepatitis delta coinfection

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Background: Hepatitis delta virus (HDV) infection accelerates the progression of liver disease in persons living with HIV and hepatitis B virus (HBV) coinfection. We explored the association between HDV infection and alanine aminotransferase (ALT) elevation during tenofovir-containing antiretroviral treatment among persons living with HIV/HBV.

Materials and methods: We included persons living with HIV/HBV with and without HDV starting tenofovir-containing antiretroviral therapy (ART) in three European cohorts with at least 18 months of follow-up. We defined HDV infection as a positive anti-HDV antibody test. We assessed risk factors for ALT elevation $\geq$ 1.25x upper limit of normal after 5 years of tenofovir-treatment using multivariate logistic regression models. The difference in ALT trends between individuals with and without HDV was evaluated using linear mixed effects models.

Results: 61/518 (11.8%) participants had an HDV infection. Among individuals with HDV, 63.9% had ALT elevation after 2 years and 55.6% after 5 years

of tenofovir, whereas the estimates were 34.1% after two and 27.0% after 5 years in those without HDV. HDV coinfection (adjusted odds ratio 2.8, 95% confidence interval 1.4–5.8) and obesity at baseline (adjusted odds ratio 3.2, 95% confidence interval 1.2–8.0) were associated with ALT elevation after 5 years of tenofovir therapy. Mean ALT levels were consistently higher during follow-up in participants with HDV compared to those without HDV.

Conclusion: Persistent ALT elevation is common in persons living with HIV/HBV in Europe despite adequate HBV therapy. HDV coinfection and obesity are independent risk factors for persistent ALT elevation during long-term tenofovir treatment.

KEYWORDS

hepatitis D (delta) virus, hepatitis B virus, HIV, coinfection, tenofovir, alanine aminotransferase elevation

Introduction

Hepatitis B virus (HBV) infection is a major cause of morbidity and mortality among persons living with HIV (PLWH) (1). Of the approximately 38 million PLWH, an estimated 8% are also living with hepatitis B (2, 3). Hepatitis delta (HDV) coinfection occurs in approximately 15% of persons living with HIV/HBV in Europe and the majority of them have detectable HDV ribonucleic acid (RNA) (4). Currently, a large majority of persons living with HIV/HBV/HDV or HIV/HBV are treated with tenofovir disoproxil fumarate (TDF) or tenofovir alafenamide (TAF) as part of their antiretroviral treatment (ART). This treatment suppresses HBV viral load successfully but the risk of liver inflammation, liver-related events and death remains elevated (5). In a recent analysis from the Swiss HIV Cohort Study, the risk of liver-related death was eight times higher among individuals with HDV infection compared to those without (6).

Tenofovir leads to liver fibrosis regression, and early alanine aminotransferase (ALT) normalization after initiation of HBV treatment is associated with lower risk for hepatocellular carcinoma in persons living with hepatitis B (7, 8). In PLWH, the effect of tenofovir on liver fibrosis regression appears to be smaller and ALT elevation was identified as an independent risk factor for advanced fibrosis (9). Data on long-term trends of ALT levels among large populations of persons living with HIV/HBV are scarce. Understanding the risk factors leading to persistent ALT elevation despite adequate HBV therapy including the impact of HDV could help reduce the risk for liver-related events in this population.

The objective of this study was to explore the association between HDV infection and long-term trends in ALT levels after initiation of tenofovir-containing ART in the Euro-B study, a multi-cohort collaboration including persons living with HIV/HBV and with HIV/HBV/HDV from the Swiss HIV Cohort Study (10), the EuroSIDA Study (11), and the French HIV/HBV cohort (12).

Materials and methods

Study design and population

We included all PLWH aged 18 years or older with two positive HBsAg measurements ≥ 6 months apart who started a tenofovir disoproxil fumarate or tenofovir alafenamide-containing ART between November 2001 and September 2019 and had at least two available ALT measurements, one at the start and the other 24 months after start of TDF or TAF treatment. We excluded participants without known HDV serology. Participants could switch from TDF to TAF or vice versa during follow-up. Detailed information on demographical, clinical, and laboratory data were collected according to the standardized study protocols of the Swiss HIV Cohort Study, the EuroSIDA Study and the French HIV/HBV cohort (10–12). Local ethical committees approved the cohort studies and written consent was obtained from all participants according to local regulations.

Outcomes and definitions

Our primary outcome was the proportion of participants with an ALT elevation ≥ 1.25x upper limit of normal (ULN) 2 and 5 years after start of tenofovir treatment in persons with and without HDV coinfection. Our secondary outcome was the difference in mean ALT levels from tenofovir start to 5 years thereafter. We defined ALT ULN as 35 international units

per liter (IU/L) for men and 25 IU/L for women according to the AASLD definition (13). We defined mild ALT elevation as ALT ≥ 1.25x to < 2.5x ULN, moderate ALT elevation as ≥ 2.5x to < 5x ULN, severe ALT elevation as ALT ≥ 5x to < 10x ULN and life-threatening ALT elevation as ≥ 10x ULN as proposed by the National Institutes of Health's Division of AIDS (14).

We classified participants with a positive anti-hepatitis delta antibody (anti-HDV) test at any time point as having HDV coinfection. We defined HBV viral load detection limit as 20 international units per milliliter (IU/ml) or the detection limit reported. Participants were considered to be hepatitis C virus (HCV) RNA positive if HCV RNA was quantifiable before tenofovir start. We defined liver cirrhosis primarily according to results from liver biopsy. If no liver biopsy was performed, we used a liver stiffness measurement > 11 kilopascal (kPa) using transient elastography or aspartate aminotransferase (AST)-to-platelet ratio (APRI) index > 2 to classify participants (15, 16). We considered reporting of ascites, bleeding from gastric esophageal varices, portal hypertension, hepatic encephalopathy, spontaneous bacterial peritonitis, and histologically confirmed diagnosis of cirrhosis, hepatorenal syndrome and liver transplantation as liver-related events. As alcohol consumption was not uniformly assessed across all cohorts, we harmonized the data and used an intake of > 25 alcohol containing units per week for men and > 20 alcohol containing units per week for women to define unhealthy alcohol use. We defined diabetes mellitus as reported diagnosis of diabetes mellitus or treatment with a blood glucose lowering drug; hypertension as reported diagnosis of arterial hypertension or treatment with an antihypertensive drug; and dyslipidemia as a total cholesterol to HDL-cholesterol ratio > 5 or treatment with a lipid lowering drug.

Statistical analysis

We defined baseline as the start date of the first tenofovir-containing ART. For the assessment of the proportion of participants with ALT elevation after two and 5 years of tenofovir-containing ART, we considered the closest measurements to baseline (−12/ + 6 months), to 24 months (± 6 months), and to 60 months (± 6 months) of tenofovir treatment. For the longitudinal assessment of ALT levels, all available ALT measurements from the closest laboratory measurement to baseline (−12/ + 6 months) up to 60 months (+ 6 months) afterward were considered. Follow-up was censored at death, loss to follow-up, last follow-up visit or 6 months after cessation of the last tenofovir-containing drug, whichever happened first. Participants interrupting tenofovir treatment were allowed to continue follow-up if they resumed treatment later on.

We compared demographic and clinical characteristics at baseline between participants with and without HDV using Pearson's chi-squared tests for categorical variables and Wilcoxon rank-sum tests for continuous variables. We assessed the proportion of participants with at least mild ALT elevation after 2 and 5 years. We used multivariable logistic regression to analyze potential risk factors for ALT elevation after two and after 5 years of tenofovir treatment. For the multivariable model, we included all variables with a p-value < 0.1 in univariable analyses, but excluded mode of HIV acquisition due to collinearity with HDV status.

We modeled mean ALT values with 95% confidence intervals (CI) over time using multivariable linear mixed effect models with a random intercept for individuals and a random slope for individual follow-up time. We included HDV status as a covariate to compare mean ALT levels between participants with and without HDV. We incorporated follow-up time as restricted cubic splines with four knots located at the 5th, 35th, 65th, and 95th percentile. We based 95% CI calculation on standard errors calculated using the delta method. We adjusted our multivariable model for sex to control for biological differences in ALT levels between males and females, and for ART experience at tenofovir start to control for potential immune reconstitution-induced hepatic flares. Treatment with TDF and TAF were included as separate time-updated covariates with an interaction term between them to take into account treatment interruptions and the potential additional beneficial impact of TAF on ALT values (17). In addition, we included all baseline variables with a p-value < 0.1 in univariable analyses of risk factors for ALT elevation after 2 and 5 years of tenofovir treatment in a preliminary model but excluded those with a p-value > 0.1 in a backward stepwise fashion from the final model. In the final model, BMI was included as a time-updated covariate rather than BMI at baseline to control for the influence of weight changes on ALT levels over time. Missing BMI assessments at a specific data point were handled by carrying the last observation forward. Missing values of categorical baseline covariates were included as a separate category. In a sub-analysis, we investigated the impact of HBV-active nucleoside reverse transcriptase inhibitor (NRTI) pretreatment on ALT levels in participants with and without HDV.

In sensitivity analyses, we ran the multivariable models using detectable HDV RNA at any time point instead of a positive anti-HDV test as the definition of HDV infection. Statistical significance was defined as a two-sided p-value < 0.05. We performed all analyses using Stata/MP 16.1 (StataCorp, College Station, TX, United States).

Results

Study population

We identified 614 participants with chronic hepatitis B starting TDF or TAF, of whom we excluded 35 without available

ALT measurements at tenofovir start and after 24 months. We further excluded 61 participants with unknown HDV serology. In total, we included 518 participants with a median follow-up time of 9.1 years [interquartile range (IQR) 5.6–13.3] after initiation of the first tenofovir-containing regimen. Excluded participants did not differ significantly from the included study population with regards to age, BMI, mode of HIV acquisition, liver cirrhosis, and hepatitis B e antigen (HBeAg) status, but they were less likely to be treated with an HBV-active NRTI prior to the initiation of tenofovir [55/96 (57.3%) vs. 387/518 (74.7%), $p < 0.001$].

Hepatitis delta virus (HDV) serology was positive in 61 (11.8%) participants. The characteristics of participants with and without HDV coinfection at start of tenofovir therapy are shown in Table 1. Participants with HDV coinfection were more likely to have acquired HIV through injection drug use (62.3% vs. 5.9%, $p < 0.001$), to have HCV replication (25.9% vs. 4.1%, $p < 0.001$), to be of European origin (82.0% vs. 65.1%, $p = 0.01$) and to have liver cirrhosis (29.4% vs. 11.0%, $p = 0.002$), but were less likely to have a detectable HBV viral load (55.6% vs. 75.9%, $p = 0.003$), and to be HBeAg-positive (31.0% vs. 54.3%, $p = 0.004$). Of 42 participants with HDV coinfection and available HDV viral load quantification, 26 (61.9%) had detectable HDV RNA. Median HDV viral load was 11,930,000 copies/ml (IQR 170,284 to 129,862,224) among participants with detectable HDV RNA and HDV genotyping was available in 18 of them with HDV genotype 1 being predominant (94.4%).

425/518 (82.0%) participants were followed for at least 5 years on tenofovir treatment and 401 (94.4%) of them had an available ALT measurement after 60 months. Of the 93 participants with less than 5 years of follow-up on tenofovir, 71 participants reached their last follow-up visit earlier or were lost to follow-up, 14 participants died and eight stopped tenofovir treatment permanently. Within the 5°year follow-up period, 15/401 (3.7%) participants had switched from TDF to TAF and 87/401 (21.7%) had interrupted tenofovir

TABLE 1 Characteristics of Euro-B participants at start of tenofovir-containing antiretroviral therapy (ART), by anti-hepatitis delta antibody (anti-HDV) status.

	anti-HDV negative $N = 457$	anti-HDV positive $N = 61$	*p*-value
Median age in years (IQR)	41 (36–47)	40 (34–44)	0.08
Median calendar year of tenofovir start (IQR)	2005 (2003–2008)	2005 (2003–2007)	0.73
Median follow-up time in years (IQR)	9.1 (5.6–13.1)	10.0 (5.6–15.0)	0.47
Female sex	81/457 (17.7%)	17/61 (27.9%)	0.06
Mode of HIV acquisition			< 0.001
men who have sex with men	256/457 (56.0%)	8/61 (13.1%)	
heterosexual	119/457 (26.0%)	13/61 (21.3%)	
injection drug use	27/457 (5.9%)	38/61 (62.3%)	
other or unknown	55/457 (12.0%)	2/61 (3.3%)	
European origin	293/450 (65.1%)	50/61 (82.0%)	0.01
CDC stage C	122/457 (26.7%)	18/61 (29.5%)	0.64
Liver cirrhosis	38/344 (11.0%)	10/34 (29.4%)	0.002
Ever reported unhealthy alcohol use	101/436 (23.2%)	19/57 (33.3%)	0.09
Diabetes mellitus	15/457 (3.3%)	0/61 (0.0%)	0.15
Hypertension	59/457 (12.9%)	8/61 (13.1%)	0.96
Dyslipidemia	181/432 (41.9%)	24/60 (40.0%)	0.78
BMI $\geq$ 30 kg/m^2	27/432 (6.3%)	2/59 (3.4%)	0.38
ART-experienced	293/457 (64.1%)	36/61 (59.0%)	0.44
Pretreatment with HBV-active NRTI*	341/457 (74.6%)	46/61 (75.4%)	0.89
ALT $\geq$ 1.25x ULN	227/457 (49.7%)	39/61 (63.9%)	0.04
Detectable HBV viral load	289/381 (75.9%)	25/45 (55.6%)	0.003
HBeAg positive	185/341 (54.3%)	13/42 (31.0%)	0.004
CD4 $\geq$ 500 cells/μl	128/455 (28.1%)	10/61 (16.4%)	0.05
Detectable HIV viral load	231/453 (51.0%)	33/61 (54.1%)	0.65
Hepatitis C RNA positive	17/416 (4.1%)	14/54 (25.9%)	< 0.001
HDV RNA positive	–	26/42 (61.9%)	–

Data are presented as median (IQR) for continuous measures, and n/total (%) for categorical measures. *424 participants received lamivudine and 1 received entecavir prior to tenofovir. 17 participants who received lamivudine received also adefovir and 2 entecavir prior to tenofovir. ALT, alanine aminotransferase; anti-HDV, anti-hepatitis delta antibodies; ART, antiretroviral therapy; BMI, body mass index; CDC, centers for disease control and prevention; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HIV, human immunodeficiency virus; IQR, interquartile range; NRTI, nucleoside reverse transcriptase inhibitors; ULN, upper limit of normal; RNA, ribonucleic acid.

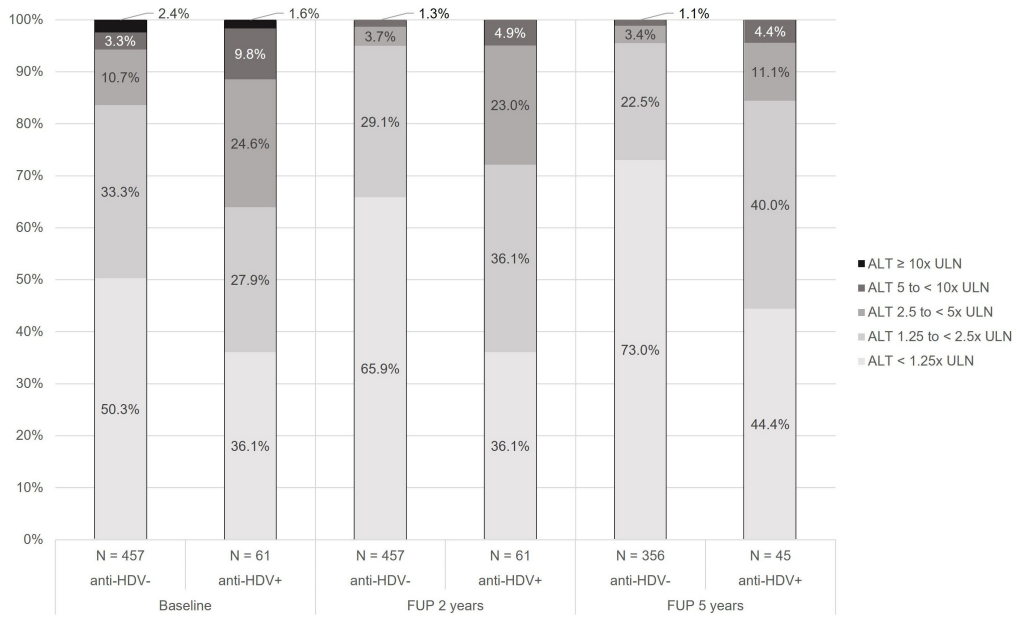

FIGURE 1
Grade of alanine aminotransferase (ALT) elevation at start, after 2 years and after 5 years of tenofovir treatment by anti-hepatitis delta antibody (anti-HDV) status. Grading according to the National Institutes of Health's Division of acquired immunodeficiency syndrome (AIDS) (14). ALT, alanine aminotransferase; anti-HDV, anti-hepatitis delta antibodies; FUP, follow-up; ULN, upper limit of normal.

treatment for > 90 days cumulatively. Proportions of treatment interruptions did not differ between HDV negative and HDV positive participants. 12/401 (3.0%) participants received treatment for HCV.

Alanine aminotransferase elevation during five years of tenofovir treatment

At start of tenofovir therapy, 227/457 (49.7%) HDV-negative participants had at least mildly elevated ALT, compared to 39/61 (63.9%) HDV-positive participants (p = 0.04). 26 (5.7%) participants without HDV had severe or life-threatening ALT elevation at start of tenofovir treatment compared to 7 (11.5%) with HDV coinfection (Figure 1). A sensitivity analysis classifying HDV coinfection as having detectable HDV RNA showed similar results with an even higher proportion of participants with at least mild ALT elevation in the group with detectable HDV RNA (84.6 vs. 49.3%, p < 0.001) (Supplementary Table 1). After 5 years of treatment, the proportion of participants with at least mildly elevated ALT decreased to 96/356 (27.0%) of HDV negative participants and 25/45 (55.6%) of the participants with HDV coinfection. Severe ALT elevation was observed in 4/356 (1.1%) HDV negative and 2/45 (4.4%) HDV positive participants (Figure 1).

In multivariable analyses, HDV coinfection was associated with ALT elevation after two [adjusted odds ratio (aOR) 5.6, 95% CI 2.6–12.4] and 5 years (aOR 2.8, 95% CI 1.4–5.8) (Table 2 and Supplementary Table 2). ALT at baseline (aOR 2.3, 95% CI 1.4–3.8), younger age (aOR 1.0, 95% CI 0.9–1.0), and obesity at baseline (aOR 3.2, 95% CI 1.2–8.0) were significantly associated with elevated ALT after 5 years of tenofovir treatment (Table 2). In a sensitivity analysis using HDV RNA instead of anti-HDV to define HDV coinfection, the multivariable models showed similar results compared to the main model: HDV RNA was strongly associated with at least mild ALT elevation after two (aOR 13.2, 95% CI 2.9–59.7) and 5 years (aOR 4.2, 95% CI 1.4–12.5) (Supplementary Table 3).

Hepatitis delta virus-positive individuals were less likely to have a detectable HBV viral load compared to HDV-negative individuals at baseline [25/45 (55.6%) vs. 289/381 (75.9%), p = 0.003], and after 5 years of follow-up [0/18 (0.0%) vs. 25/204 (12.3%), p = 0.11]. Of note, HDV-negative participants with replicating HBV infection 5 years after tenofovir start were more likely to have elevated ALT compared to those with suppressed HBV viral load [12/50 (24%) vs. 13/154 (8.4%), p = 0.004].

Longitudinal analysis of alanine aminotransferase levels over five years of tenofovir treatment

Alanine aminotransferase trends were assessed among 510 participants and included a total of 6,687 ALT measurements (median 11 measurements per participant, IQR 8–15). The difference in predicted mean ALT values between participants

TABLE 2 Risk factors at start of tenofovir-containing antiretroviral therapy (ART) for alanine aminotransferase (ALT) elevation (≥ 1.25x ULN) after 5 years of tenofovir treatment.

	Unadjusted		Adjusted	
	OR (95% CI)	*P*-value	OR (95% CI)[†]	*P*-value
Anti-HDV status				
negative	1.0	(ref)	1.0	(ref)
positive	3.4 (1.8–6.4)	< 0.001	2.8 (1.4–5.8)	0.005
ALT at baseline				
< 1.25x ULN	1.0	(ref)	1.0	(ref)
≥ 1.25x ULN	2.4 (1.5–3.7)	< 0.001	2.3 (1.4–3.8)	0.001
Age [years]	1.0 (0.9–1.0)	0.01	1.0 (0.9–1.0)	0.02
Female sex	1.2 (0.7–2.0)	0.54		
Mode of HIV acquisition		0.005		
men who have sex with men	1.0			
heterosexual	1.0 (0.6–1.8)			
injection drug use	3.1 (1.6–5.9)			
other or unknown	1.0 (0.5–2.2)			
Liver cirrhosis	1.7 (0.8–3.5)	0.14		
History of liver related event	2.4 (1.0–5.8)	0.04	2.1 (0.7–5.9)	0.18
Ever reported unhealthy alcohol use	1.2 (0.8–2.0)	0.41		
Dyslipidemia	1.2 (0.8–1.8)	0.50		
Diabetes mellitus	1.3 (0.4–4.6)	0.65		
Hypertension	1.2 (0.6–2.2)	0.59		
BMI ≥ 30 kg/m^2	2.4 (1.0–5.7)	0.05	3.2 (1.2–8.0)	0.02
HBV-active NRTI pretreatment	1.1 (0.7–1.8)	0.70		
ART-experienced	1.4 (0.9–2.1)	0.19		
Detectable HBV viral load	1.0 (0.6–1.7)	0.96		
HCV RNA positive	2.4 (1.0–5.6)	0.05	1.4 (0.5–3.8)	0.47

[†]347 participants included in complete case analysis. ALT, alanine aminotransferase; anti-HDV, anti-hepatitis delta antibodies; ART, antiretroviral therapy; BMI, body mass index; CI, confidence interval; FUP, follow-up; HBV, hepatitis B virus; HCV, hepatitis C virus; NRTI, nucleoside reverse transcriptase inhibitors; OR, odds ratio; RNA, ribonucleic acid.

with and without HDV coinfection was + 17 IU/L (95% CI 5–29) at baseline, + 20 IU/L (95% CI 10–29) after 2 years and + 15 IU/L (95% CI 0–31) after 5 years of tenofovir treatment (**Figure 2**). In a sensitivity analysis using HDV RNA instead of anti-HDV to define HDV coinfection, the difference in mean ALT values between participants with and without HDV coinfection increased to + 29 IU/L (95% CI 12–46) at baseline, + 31 IU/L (95% CI 18–44) after 2 years and + 40 (95% CI 18–62) after 5 years of tenofovir treatment in the multivariable model (**Supplementary Figure 1**). HBV-active NRTI treatment before the initiation of tenofovir treatment was not associated with ALT trends in participants with and without HDV coinfection (**Supplementary Figure 2**).

Discussion

In our multi-cohort study of persons living with HIV/HBV in Europe, over 30% of study participants had elevated ALT levels after 5 years of tenofovir therapy, this risk being three times higher in persons living with HDV coinfection. Younger age, obesity and ALT levels at the start of tenofovir therapy were also associated with ALT elevation after 5 years. Our study highlights the need to identify and address risk factors for persistent liver inflammation among persons living with HIV/HBV, particularly in those with HDV coinfection.

During 5 years of tenofovir therapy, ALT levels were persistently higher among persons with HDV coinfection compared to those without HDV. In the subgroup of participants with quantifiable HDV RNA, the difference in ALT levels compared to HDV negative individuals was even more accentuated. Our findings correspond with the results of a recent study in HIV-negative individuals with HBV treatment in Taiwan, in which HDV RNA positivity was found to be the strongest factor associated with ALT elevation after 2 years of NRTI therapy (18). ALT elevation in HDV positive individuals during treatment could be explained by the marginal impact of tenofovir on HDV replication, despite its efficacy in suppressing HBV viral load (19, 20). Persistent HDV replication contributes to chronic liver inflammation

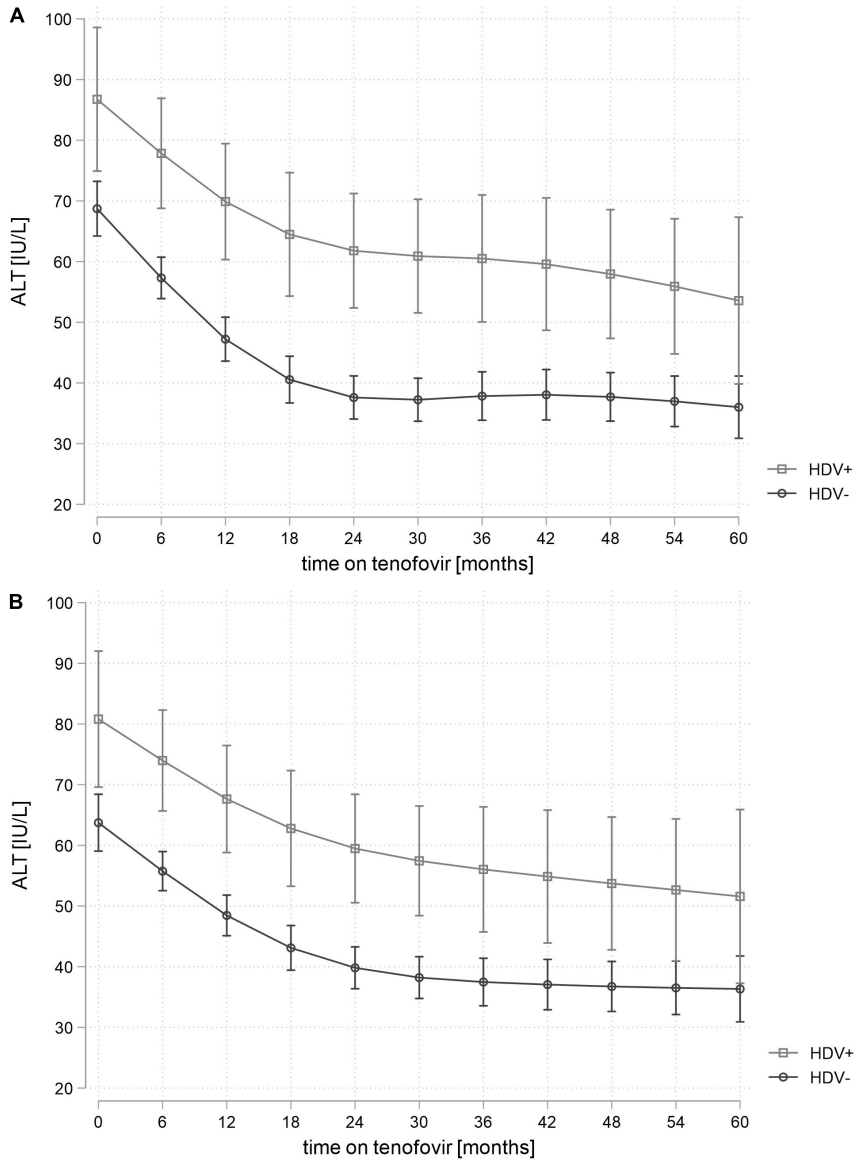

FIGURE 2
Unadjusted **(A)** and adjusted* **(B)** predicted mean alanine aminotransferase (ALT) values in participants with and without hepatitis delta virus (HDV) coinfection during treatment with tenofovir. Adjusted for ALT level, age, sex, detectable hepatitis B virus (HBV) viral load, hepatitis C virus (HCV) ribonucleic acid (RNA) status and antiretroviral therapy (ART)-experience at baseline, time-updated body mass index (BMI) and treatment with tenofovir prodrugs (tenofovir disoproxil fumarate or tenofovir alafenamide). ALT, alanine aminotransferase; ART, antiretroviral therapy; BMI, body mass index; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV+, anti-hepatitis delta antibodies positive; HDV-, anti-hepatitis delta antibodies negative; IU/L, international units per liter; RNA, ribonucleic acid.

and leads to high rates of liver decompensation and death in persons living with HIV/HBV/HDV (21). A recent study in persons living with HIV/HBV from Italy observed a doubling of the risk for a composite outcome of liver-related events, liver-related death, and non-invasive assessment of cirrhosis in individuals with HDV coinfection (22). Currently, HDV treatment options remain limited for these patients, despite the recent approval of bulevirtide (23). Our study demonstrates that persistent liver inflammation occurs in a substantial number of PLWH with HDV despite optimal HBV therapy, which underscores the need for HDV testing and liver disease monitoring in all persons living with chronic hepatitis B (24, 25).

We found higher rates of ALT elevation in HDV-negative persons living with HIV/HBV compared to published data from HIV-negative persons with HBV: in an analysis of 471 individuals from Europe, North America, Australia, and New-Zealand who participated in two randomized controlled trials

initially assessing the antiviral efficacy of TDF in comparison to adefovir, less than 20% had ALT elevation after 5 years of TDF therapy (26). However, comparison across studies is limited by the differences in clinical and sociodemographic characteristics, as well as in treatment eligibility criteria in the presence of HIV infection (24, 25). A recent study among adults living with HIV and HBV found histologic evidence of fatty liver disease in 30% of persons, which was associated with elevated ALT over time (27). In our study, the presence of obesity increased the risk for ALT elevation after 5 years of tenofovir therapy. ALT elevation seems to be common among persons living with HIV and HBV in absence of HDV coinfection, which highlights the need to address and appropriately treat metabolic risk factors for liver inflammation and fibrosis among all PLWH (24).

Our study provides detailed information on ALT levels over time from a large cohort of persons living with HIV and HBV across Europe. Our strict inclusion criteria and comprehensive clinical and virological data allowed us to obtain robust estimates for the association between HDV infection and liver inflammation. With detailed treatment histories available, we were able to disentangle the impact of HBV treatment prior to tenofovir, ART as well as metabolic and infectious comorbidities on ALT levels. However, given the limited number of participants treated with TAF in our study, we were not able to assess if long-term ALT trends depended on the type of tenofovir prodrug used (28). Hepatitis serologies and data on medical history like alcohol consumption were assessed according to the specific protocols of the participating cohorts, and were not always collected uniformly. Furthermore, some data on covariates were missing, as depicted in **Table 1**. However, the bias introduced should be small as the amount of missing values was similar in HDV positive and negative participants except for the assessment of liver cirrhosis. As HDV status was not assessed systematically at start of tenofovir therapy and serial HDV assessments were not available, HDV-positive individuals may have been at different stages of HDV infection at start of tenofovir therapy. In addition, we cannot differentiate participants living with HIV/HBV/HDV at start of tenofovir therapy from those acquiring HDV as superinfection during the study period. This could have led to an underestimation of the difference in ALT levels in case of a participant classified as HDV negative newly acquiring HDV after starting tenofovir therapy.

In summary, coinfection with hepatitis delta was an independent risk factor for persistent ALT elevation during long-term tenofovir treatment in persons living with HIV/HBV. Furthermore, obesity was independently associated with higher ALT levels over time. Careful monitoring of ALT elevations and liver disease progression is recommended in persons living with HIV/HBV, particularly in those with HDV coinfection or other comorbidities leading to liver inflammation.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: For open data sharing, the data is too dense and comprehensive to preserve patient privacy in persons living with HIV. The participating cohorts can be approached for data requests. Requests to access these datasets should be directed to http://www.shcs.ch/contact for the Swiss HIV Cohort Study and eurosida.rigshospitalet@regionh.dk for EuroSIDA.

Ethics statement

The studies involving human participants were reviewed and approved by Kantonale Ethikkommission Bern and other national ethical committees from the different cohort sites (for the Swiss HIV Cohort Study: https://shcs.ch/206-ethic-committee-approval-and-informed-consent, for EuroSIDA: https://chip.dk/Research/Studies/EuroSIDA/Study-documents). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LB, CB, GW, and AR conceived the study. LB analyzed the data. LB, AB, GW, and AR wrote the first draft of the manuscript. All authors collected and provided data for the study, reviewed and commented on the draft, and approved the final version.

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Conflict of interest

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Supplementary material

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Acute and chronic HBV infection in central Argentina: High frequency of sub-genotype F1b, low detection of clinically relevant mutations and first evidence of HDV

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Introduction: Genomic analysis of hepatitis B virus (HBV) identifies phylogenetic variants, which may lead to distinct biological and clinical behaviors. The satellite hepatitis D virus (HDV) may also influence clinical outcomes in patients with hepatitis B. The aim of this study was to investigate HBV genetic variants, including clinically relevant mutations, and HDV infection in acute and chronic hepatitis B patients in central Argentina.

Methods: A total of 217 adult HBV infected patients [acute (AHB): n = 79; chronic (CHB): n = 138] were studied; 67 were HBV/human immunodeficiency virus (HIV) coinfected. Clinical and demographic data were obtained from medical records. Serological markers were determined. Molecular detection of HBV and HDV was carried out by RT-Nested PCR, followed by sequencing and phylogenetic analysis.

Results: Overall, genotype (gt) F [sub-genotype (sgt) F1b] was the most frequently found. In AHB patients, the gts/sgts found were: F1b (74.7%) > A2 (13.9%) > F4 (7.6%) > C (2.5%) > A1 (1.3%). Among CHB patients: F1b (39.1%) > A2 (23.9%) > F4 (18.2%) > D (9.4%) > C and F6 (3.6% each) > A1, A3 and B2 (0.7% each). The distribution of sgt A2 and gt D was significantly different between HBV mono and HBV/HIV coinfected patients [A2: 15.9% vs. 35.7% ($p < 0.05$), respectively and D: 14.6% vs. 1.8% ($p < 0.05$), respectively]. Mutation frequency in basal core promoter/pre-Core (BCP/pC) region was

35.5% (77/217) [AHB: 20.3% (16/79), CHB: 44.2% (61/138)]. In the open reading frame (ORF) S, mutations associated with vaccine escape and diagnostic failure were detected in 7.8% of the sequences (17/217) [AHB: 3.8% (3/79), CHB: 10.1% (14/138)]. ORF-P amino acid substitutions associated with antiviral resistance were detected in 3.2% of the samples (7/217) [AHB: 1.3% (1/79), CHB 4.3%, (6/138)]. The anti-HDV seropositivity was 5.2% (4/77); one sample could be sequenced, belonging to gt HDV-1 associated with sgt HBV-D3.

Discussion: We detected an increase in the circulation of genotype F in Central Argentina, particularly among AHB patients, suggesting transmission advantages over the other genotypes. A low rate of mutations was detected, especially those with antiviral resistance implications, which is an encouraging result. The evidence of HDV circulation in our region, reported for the first time, alerts the health system for its search and diagnosis.

KEYWORDS

hepatitis B virus, HBV, antiviral resistance, mutant, genotypes, Argentina

1. Introduction

Hepatitis B virus (HBV) infection is currently one of the main public health problems worldwide. This is a human pathogen that leads to both self-limited and chronic infections. Despite the availability of a safe and effective vaccine, the World Health Organization (WHO) estimates that 296 million people were living with chronic hepatitis B infection in 2019, with 1.5 million new infections each year (1).

Currently, 10 HBV genotypes (gt) (A to J), -which present >8% genetic divergence- and various sub-genotypes (sgt) -presenting >4% genetic divergence- have been described (2). Determining the viral gt, sgt and isolate is useful to understand the evolution and the epidemiology of the virus. Several clinical and epidemiological observations suggest that genetic differences in viral gts may underline differences in biological and clinical parameters (3, 4).

Changes in the molecular genotype profile over time can be due to multiple factors, in addition to those inherent to the evolutionary advantages between variants. Population movements that favor the introduction of new variants, which in turn can generate new recombinant strains, changes in cultural/social patterns that favor or hinder certain modes of transmission, global human migrations that introduce genotypes differing from those found in the original inhabitants, the most efficient antiviral treatments for some genotypes than for others, might have been involved (5, 6).

Specific mutations have been described in diverse parts of the HBV genome. Nucleotide changes in the Basal Core Promoter (BCP) and preCore (pC) regions, which are associated with the regulation and expression of hepatitis B "e" antigen (HBeAg), have been associated with more severe clinical courses (7–9). Mutations that cause a conformational change in the "a" determinant of the hepatitis B surface antigen (HBsAg) may affect protein antigenicity, essential for inducing neutralizing antibodies, and be responsible for preventing vaccine- or anti-HBV immunoglobulin-induced immunity and providing false-negative results in serological tests (10–12).

Hepatitis delta virus (HDV) is a satellite virus of HBV. Globally, nearly 5% of people who have chronic hepatitis B are HDV positive (13). Co-infection or superinfection with HDV is considered the most severe form of chronic viral hepatitis due to more rapid progression toward liver-related disease and hepatocellular carcinoma (14). Eight HDV genotypes have been identified all over the world, each of which might have a different clinical outcome (14). While HDV genotypes 1 and 3 have been associated with lower remission rates and a more adverse clinical outcome (15). There is no specific interaction between HBV and HDV genotypes, and the combination of genotypes seems to simply reflects the most common genotypes circulating in a given region (16, 17).

In Argentina, the distribution of HBV genotypes has been changing over time, and has reflected the population movements that have occurred in our territory. Genotypes F (the major genotype found), A, B, C, and D have been described, with frequencies that vary according to the geographic region and the population studied (18–27).

Few investigations have been carried out in our region regarding biological and clinical implications of the circulating genotypes and sub-genotypes. More recently, Di Lello et al. (25), found vaccine escape mutations, diagnostic failure mutations, and antiviral resistance mutations in 7.5, 10.7, and 5.1% of cases, respectively. In relation to HDV, there are very few reports. Two previous studies show HDV-1 detection in the Amerindian population of northeastern of Argentina (28) and in blood donors from the Buenos Aires province (22).

In order to deepen the studies of HBV in our country, the aim of the present study was to investigate the infecting

genotype, sub-genotype and clinically relevant mutations in acute and chronic HBV infections in the central region of Argentina. Additionally, we investigated HDV infections.

2. Materials and methods

2.1. Study population

A cross-sectional, observational, and retrospective study was conducted on 217 HBV adult infected patients determined by the presence of HBsAg, who had access to public health centers of Cordoba (the second most populated inland province of Argentina), between 2010 and 2017.

Serum samples were classified as acute hepatitis B (AHB, n = 79) or chronic hepatitis B (CHB, n = 138). Diagnostic criteria for AHB were as follows: acute onset of symptoms without a history of chronic HBV infection, levels of serum alanine aminotransferase (ALT) >10-fold the upper reference limit, positivity for IgM antibody to the hepatitis B core antigen (anti-HBc), a rapid drop of HBsAg titer, serum HBV-DNA elimination and HBeAg seroconversion at convalescent phase. The diagnosis was confirmed by HBsAg clearance within 6 months after the initial onset. CHB met the following criteria: HBsAg positivity for more than 6 months.

HIV-infected patients were included. Patients were divided into two groups: patients with acute HBV infection (AHB: HBV + / HIV-, n = 68 and HBV + /HIV + , n = 11) and chronic patients (CHB: HBV + / HIV-, n = 82 and HBV + /HIV + , n = 56).

Demographic data (age, gender, and HBV viral load) were obtained from medical records. Antiretroviral therapy data were obtained from 114 chronic patients. Fibrosis score (Metavir F0-4) and inflammatory activity in liver tissue data were available from 30 chronic patients.

2.2. Serological markers of HBV infection and HBV viral load determination

The following serological markers were evaluated by chemiluminescent microparticle immunoassay (CMIA) on the ARCHITECT system (Abbott Diagnostics, USA): HBeAg; HBsAg; IgM anti-HBc; anti-HBe. HBV DNA levels were determined using the COBAS® TaqMan® HBV Test (Roche Diagnostics, Germany), targeting the highly conserved pre-Core/Core region of the HBV genome (limit of detection: 29 UI/mL). Samples with HBV viral load greater than the maximum quantification limit were diluted and reprocessed until the exact viral load value was obtained.

2.3. Anti-HDV antibody detection

Total anti-HDV antibodies were assessed in 77 HBsAg (+) samples, 3 from AHB patients and 74 from CHB patients, using the enzyme immunoassay (EIA) ETI-AB-DELTAK-2 (DiaSorin, Italy).

2.4. Molecular detection and sequencing of HBV and HDV

Nucleic acid extraction was performed using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Germany), strictly following the manufacturer's instructions. For HBV, the S gene and the BCP-pC gene regions were amplified (585 bp and 742 bp, respectively) using the protocol described by Pisano et al. (20) (**Supplementary Table 1**).

For HDV molecular detection, a reverse transcription using the ImProm-II™ Reverse Transcription System (Promega, USA), and random hexamer primers, followed by a Nested-PCR for amplification of a 353 bp genomic fragment of the HDAg was carried out (22) (**Supplementary Table 1**).

In all cases, PCR products were purified using the PureLink™ Quick Gel Extraction Kit (Invitrogen, USA). Direct nucleotide sequencing reaction in both directions was carried out using a 3500xL Genetic Analyzer (Applied Biosystems, USA), using a locally standardized and validated protocol with the same primers used in amplification stages.

2.5. Phylogenetic analysis

Phylogenetic analyses were performed using the maximum likelihood method with the software MEGA (v6.0) (29), under the appropriate nucleotide substitution model selected by jModeltest (30), according to the Akaike Information Criterion. The robustness of the reconstructed phylogenies was evaluated by bootstrap analysis (1000 replicates). For HBV, the analysis was performed by combining the sequences obtained from the S and BCP-pC genes, while for HDV, we used the sequence from the HDAg fragment.

2.6. Analyses of mutations in ORF-S, ORF-P, and BCP-pC genomic regions

The HBV nucleotide and amino acid sequences were aligned and compared with the prototype strains of each sub-genotype using the MEGA (v6.0) (29) program, the Mutation Reporter Tool (31), and Geno2pheno HBV tools from the Max Planck

Institute.[1] Amino acid sequences corresponding to the ORF-S and ORF-P genes (using sequences obtained from the S gene), as well as the ORF-pC/C (using sequences obtained from BCP-pC) were analyzed.

In order to search for the most significant HBV surface mutants, aa 99–169 within the HBsAg gene were examined. According to previous reports, 12 clinically relevant amino acid positions (118, 120, 126, 129, 130, 133, 134, 141, 142, 143, 144, and 145) were analyzed (25, 32). Positions rtL80, rtI169, rtV173, rtL180, rtA181, rtS184, rtA194, rtS202, rtM204, rtN236T, and rtM250V in the polymerase gene were investigated in order to evaluate treatment resistance mutants for the most widely used antivirals. Additionally, positions 1753, 1762, 1764, and 1896 in the BCP/pC region were studied. Mutations at these positions have been reported to modulate HBeAg expression.

2.7. Nucleotide sequence accession numbers

Nucleotide sequences obtained in this work were deposited at the GenBank database under accession numbers: HBV: OM333932 to OM334148 for the S gene and OM456810 to OM456985 for the BCP-pC genomic region; HDV: ON751779.

2.8. Statistical analysis

Statistical analyses were conducted using StataMP 14 program. The sociodemographic categorical variables that characterized the sample under study were expressed in proportions stratified by sex. Quantitative values with normal distribution are presented with mean and standard deviation. Data that do not have a normal distribution are presented with median and interquartile ranges. To identify differences between populations, a difference in proportions, a difference in means (t-student), or a difference in medians (W-Mann Whitney) was used according to the distribution of the data, which was determined by means of the normality test (Shapiro–Francia). A level of significance equal to 0.05 was adopted. The strength of the relationship was estimated by using Odds Ratio (CI 95%).

2.9. Ethical aspects

This work was evaluated and approved by the Institutional Ethics Committee for Child and Adult Health Research of the Ministry of Health of Córdoba province, Argentina (RePIS N° 2701).

1 http://hbv.bioinf.mpi-inf.mpg.de/

3. Results

3.1. Clinical and epidemiological characteristics of the studied population

The median age of the population analyzed was 41 years (range 18–73 years) and the 70.5% (153/217) were male. Seventy-nine cases were classified as AHB [(36.4%), mean age 41.7 (± 11.4) years, 67.1% male] and 138 as CHB [(63.6%), mean age 41.3 (± 11.5) years, 66.7% male]. Among AHB individuals, 11 were also human immunodeficiency virus (HIV) (+) [(13.9%), mean age 36.4 (± 9.8) years, 63.6% male] and among CHB, 56 were HBV/HIV co-infected [(40.6%), mean age 38.1 (± 9.7) years, 94.6% male] (Table 1).

Overall, among patients with chronic infection 45.6% (63/138) received antiviral treatment. Among mono-infected patients 30.5% (25/82) were under treatment [9 with entecavir, 9 with tenofovir, 7 changed their treatment regimen to tenofovir after receiving interferon (n = 2), lamivudine/3TC (n = 2) and entecavir (n = 3)], 45.1% (37/82) without treatment and in 24.4% (20/82) data were not available. In HBV/HIV co-infected patients, 67.8% (38/56) were on antiviral treatment regimen with tenofovir, 14.3% (8/56) did not receive therapy, and in 17.9% (10/56) data were not recorded. CHB HBV/HIV co-infected patients had a higher percentage of treatment than those monoinfected with HBV ($p < 0.05$).

In the acute stage, no significant differences in age between HBV-monoinfected patients and HBV/HIV co-infected patients were found ($p = 0.09$). Chronic HBV/HIV co-infected patients were significantly younger than CHB-monoinfected patients ($p < 0.05$). The male to female ratio showed a significant difference between CHB-monoinfected patients (1.3) and CHB-HBV/HIV co-infected patients (17.7) ($p < 0.001$).

The HBeAg positivity rate and the median HBV viral load were significatively higher in: a- AHB vs. CHB among mono-infected patients (3,80E + 05 vs. 1.49E + 03–$p < 0.001$), and b- CHB HBV/HIV co-infected patients vs. CHB mono-infected patients (3,34E + 07 vs. 1.49E + 03–$p < 0.001$) (Table 1).

In all cases, HBV viral loads were significantly higher in HBeAg-positive patients (Table 1).

3.2. Genotype and sub-genotype distribution

Phylogenetic analysis of the S and BCP/pC genomic regions allowed genotyping 100.0% of the samples and subtyping 97.2% (Figures 1, 2). The subtype could not be defined in all samples belonging to gt C (Figures 1, 2) for this reason; this genotype was considered as a whole in subsequent analysis). For gt D, 2 samples grouped within sgt D1, 7 within sgt D2 and in 4 samples the viral subtype could not be defined (Figure 2). Due to the

TABLE 1 Age, gender and hepatitis B virus (HBV) viral load distribution among different stages of HBV infection in mono and HBV/HIV co-infected patients.

Acute hepatitis B (AHB) *N* = 79						
	HBV mono-infected patients			HBV/HIV Co-infected patients		
		HBeAg (+)	HBeAg (−)/anti-HBeAg (+)		HBeAg (+)	HBeAg (−)/anti-HBeAg (+)
N	68	54[c]	14	11	10	1
Age (Mean ± SD)	42.5 ± 11.4	41.2 ± 11.1	47.0 ± 12.5	36.4 ± 9.8	37.1 ± 10.0	29.0
M:F* (M/F Ratio)	46:22 (2.1)	36:18 (2.0)	10:4 (2.5)	7:4 (1.8)	7:3 (2.3)	0:1
Viral load (UI/ml)						
Median	3.80E + 05[e]	2.41E + 06	5.48E + 03	3.80E + 07	7.44E + 07	—
Chronic hepatitis B (CHB) *N* = 138						
	HBV mono-infected patients			HBV/HIV Co-infected patients		
		HBeAg (+)	HBeAg (−)/anti-HBeAg (+)		HBeAg (+)	HBeAg (−)/anti-HBeAg (+)
N	82	20[c,d]	62	56	48[d]	8
Age (Mean ± SD)	43.5 ± 12.1[a]	43.3 ± 12.3	43.5 ± 12.1	38.1 ± 9.7[a]	37.4 ± 9.4	42.4 ± 10.1
M:F (M/F Ratio)	47:35 (1.3)[b]	16:4 (4.0)	31:31 (1.0)	53:3 (17.7)[b]	45:3 (15.0)	8:0
Viral load (UI/ml)						
Median	1.49E + 03[e,f]	1.07E + 08	7.25E + 02	3.34E + 07[f]	5.22E + 07	2.55E + 05

*M, male; F, female.
[a]Age: Chronic hepatitis B (CHB) mono-infection vs. CHB HBV/HIV co-infection $p < 0.05$.
[b]M/F ratio: CHB mono-infection vs. CHB HBV/HIV co-infection $p < 0.001$.
[c]HBeAg (+): Acute hepatitis B (AHB) mono-infection vs. CHB mono-infection $p < 0.001$.
[d]HBeAg (+): CHB mono-infection vs. CHB HBV/HIV co-infection $p < 0.001$.
[e]Median viral load: AHB mono-infection vs. CHB mono-infection $p < 0.001$.
[f]Median viral load: CHB mono-infection vs. CHB HBV/HIV co-infection $p < 0.001$.

low number of samples of each genotype D subtype, it was also considered as a whole in subsequent analysis.

The overall genotypes/sub-genotypes distribution for the study cohort was as follows: A1 (0.9%), A2 (20.3%), A3 (0.45%), B2 (0.45%), C (3.2%), D (6.0%), F1b (52.1%), F4 (14.3%) and F6 (2.3%).

3.3. Genotype distribution in AHB patients

Among AHB patients, we found the following genotypes: F (82.3%) > A (15.2%) > C (2.5%). In the same way, HBV sub-genotypes were in the following proportions: F1b (74.7%) > A2 (13.9%) > F4 (7.6%) > C (2.5%) > A1 (1.3%) (**Figure 1**). No significant differences were observed in gt/sgt distribution between HBV-mono-infected and HBV/HIV co-infected patients in the acute stage (**Figure 3A** and **Supplementary Table 2**).

No significant differences were observed among HBV viral loads among patients infected with different HBV gts, in both HBV mono-infected patients and HBV/HIV co-infected patients ($p > 0.05$) (**Supplementary Table 2**).

3.4. Genotype distribution in CHB patients

Among CHB patients the HBV genotype proportion was: F (60.9%) > A (25.4%) > D (9.4%) > C (3.6%) > B (0.7%). In the same way, HBV sub-genotypes were in the following proportions: F1b (39.1%) > A2 (24%) > F4 (18.2%) > D (9.4%) > C and F6 (3.6% each) > A1, A3 and B2 (0.7%) (**Figure 2**). Sub-genotypes distribution was different between mono- and co-infected patients (**Figure 3B**). In the 82 HBV-mono-infected sgt F1b was present in 34.1% of cases, followed by sgt F4 (23.2%) and A2 (15.9%). The gt D, sgt F6 and gt C were present in 14.6, 6.1, and 2.4% of cases, respectively. Sgt A1, A3, and B2 were observed in 1.2% of cases (**Figure 3B** and **Supplementary Table 1**). Among the 56 HBV/HIV co-infected patients, samples grouped as follows: F1b (46.4%), A2 (35.7%), F4 (10.7%), C (5.4%), and D (1.8%) (**Figure 3B** and **Supplementary Table 2**). Among the most prevalent gt/sgt, a significant difference was only found in the distribution between HBV-mono-infected and HBV/HIV-co-infected for sgt A2 (15.9 vs. 35.7%, $p < 0.05$) and for gt D (14.6% vs. 1.8%, $p < 0.05$) (**Figure 3B** and **Supplementary Table 2**).

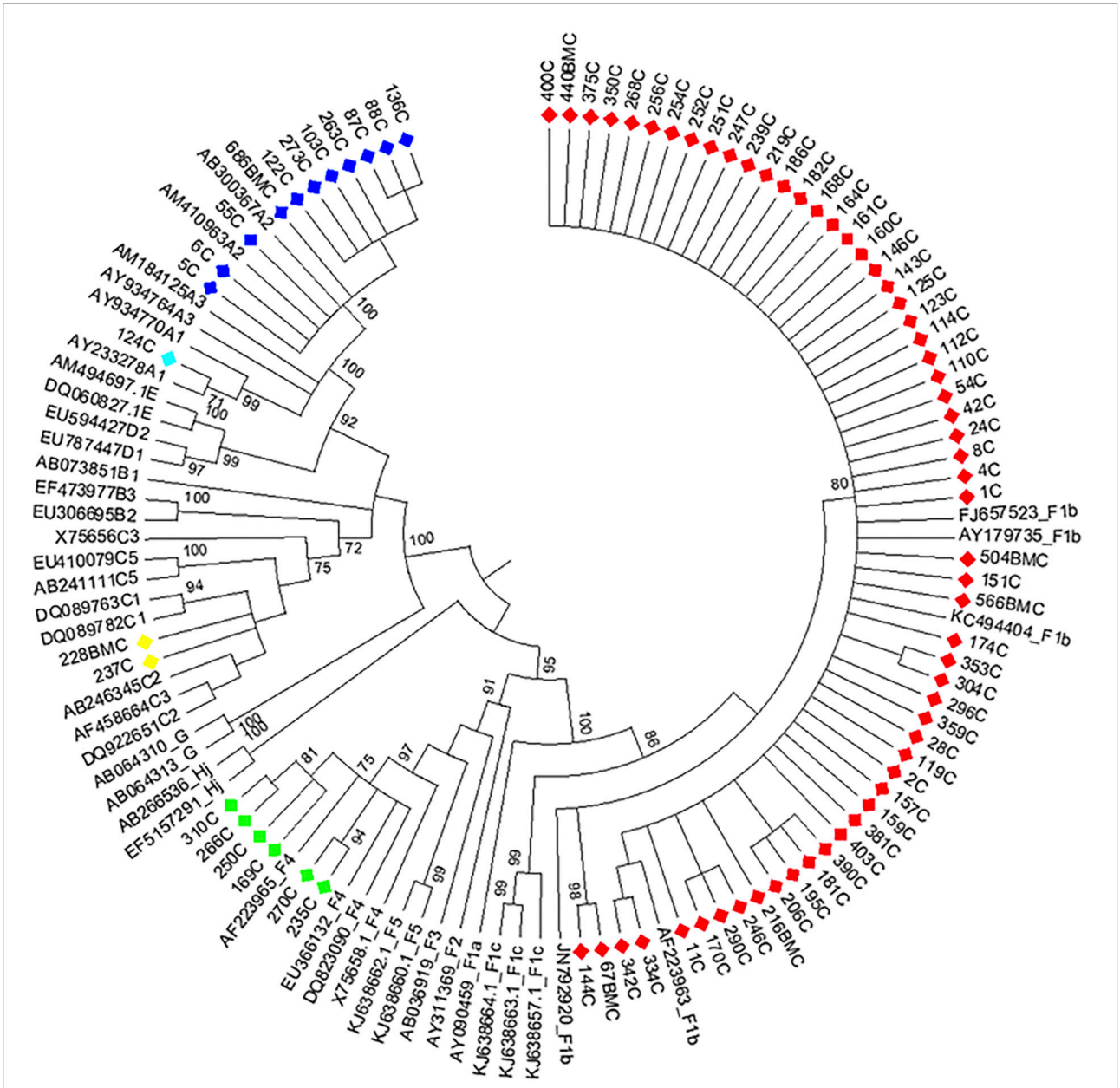

FIGURE 1
Maximum likelihood phylogeny of acute hepatitis B samples from Córdoba, Argentina. Maximum likelihood phylogenetic tree obtained using sequences of 1,169 nucleotides, resulting from concatenated sequences of the S (471 nucleotides) and BCP-pC genomic regions (698 nucleotides) of acute hepatitis B samples from central Argentina and reference sequences from each genotype or subgenotype available at GenBank. The phylogenetic tree was constructed with MEGA v.6 (bootstrap: 1000 replicates). References: A1, light blue; A2, blue; C, yellow; F1b, red; F4, light green.

No significant differences were observed among HBV viral loads among patients infected with different HBV gts, in both, HBV mono-infected patients as well as HBV/HIV co-infected patients ($p > 0.05$) (**Supplementary Table 2**). However, statistically significant differences were only observed in mono-infected CHB patients between genotypes A and D (7.37E + 03 vs. 5.11E + 02–$p < 0.05$).

3.5. Comparison between genotype distribution among AHB and CHB patients

Although sgt A2 was more prevalent among CHB patients compared to AHB patients, no significant differences between the two groups were found (**Figures 3A, B** and

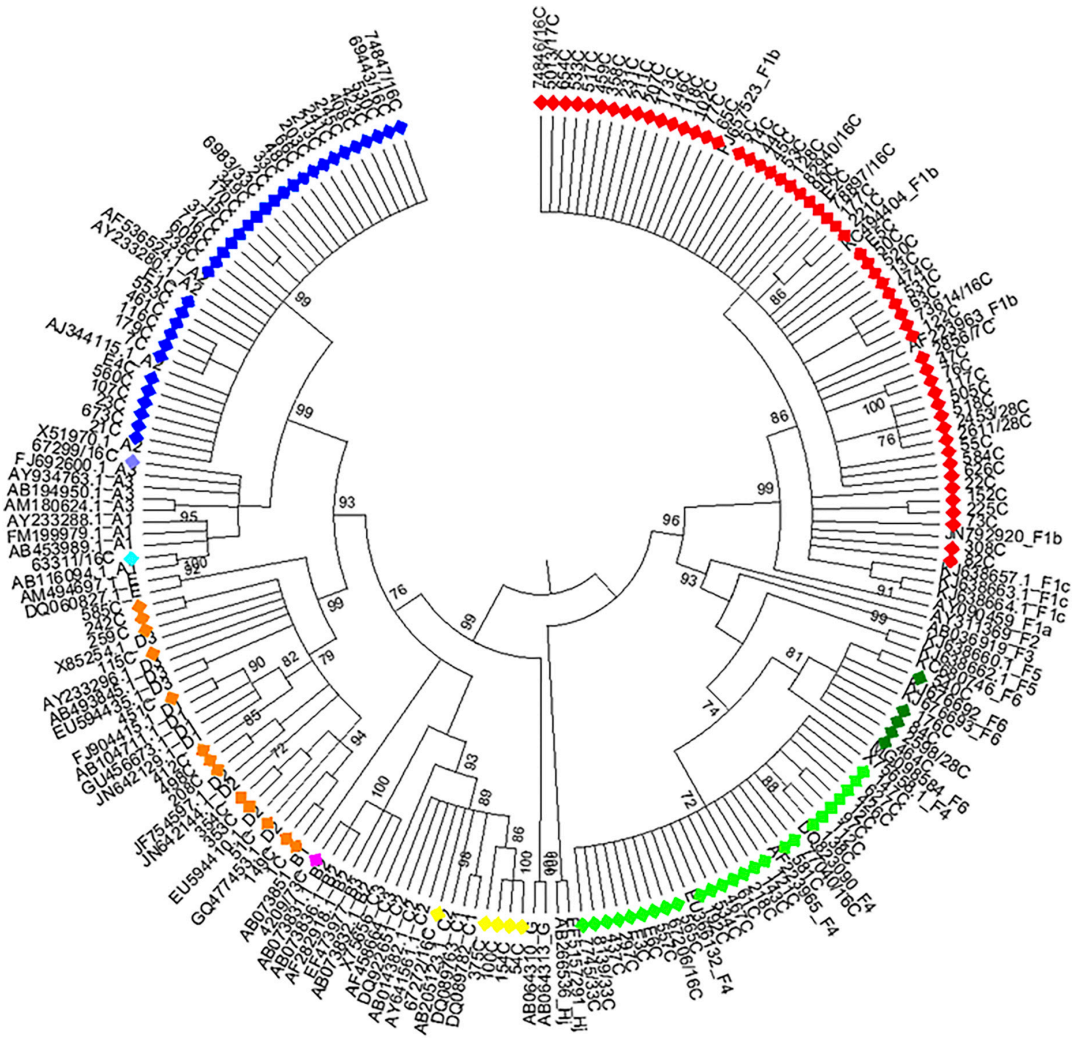

FIGURE 2
Maximum likelihood phylogeny of chronic hepatitis B samples from Córdoba, Argentina. Maximum likelihood phylogenetic tree obtained using sequences of 1,169 nucleotides, resulting from concatenated sequences of the S (471 nucleotides) and BCP-pC genomic regions (698 nucleotides) of chronic hepatitis B samples from central Argentina and reference sequences from each genotype or subgenotype available at GenBank. The phylogenetic tree was constructed with MEGA v.6 (bootstrap: 1000 replicates). References: A1, light blue; A2, blue; A3, lavender; B, fuchsia; C, yellow; D, orange; F1b, red; F4, light green; F6, green.

Supplementary Table 1). The distribution of sub-genotypes F was significantly different exclusively between AHB and CHB mono-infected patients. A higher detection frequency of F1b (74.7%) was found in AHB than in CHB (39.1%) ($p < 0.001$), and lower F4 (7.6%) frequency in AHB than in CHB (18.1%) ($p < 0.05$). Sgt F6 and gt D were only described in CHB patients (Figures 3A, B and Supplementary Table 2).

3.6. Liver injury and genotype distribution among CHB

In 30 patients with CHB infection and available liver tissue [66.7% (20/30) male, mean age 41.8 (± 12.0) years, 20.0% (6/30) HBV/HIV co-infected], the degree of fibrosis (F0-4) was recorded. Nine of them registered grade > F2 (median HBV viral load = 7.9 log10 UI/mL), seven had grade F1 (median HBV viral load = 4.1 log10 IU/mL), and the remaining patients registered stage F0 (median HBV viral load = 3.1 Log10 IU/ml). Five patients presented elevated transaminase levels, all with liver fibrosis stages F3-F4 (Supplementary Table 3).

The general distribution of genotypes among these patients was: F (53.3%) > A (30.0%) > D (13.3%) > C (3.3%). In the group of subjects with liver fibrosis > F2, the genotypes found were: A (55.6%) > F (44.4%), while among the patients with liver fibrosis F0 and F1, genotypes were: F (57.2%) > D (19.0%) = A (19.0%) > C (4.8%).

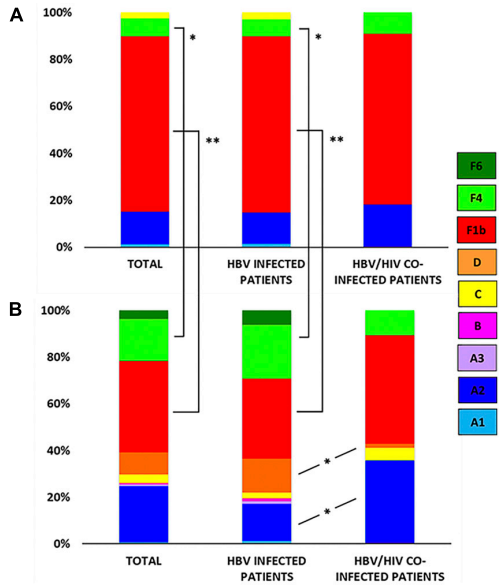

FIGURE 3
Genotype/sub-genotype distribution in acute hepatitis B (AHB) and chronic hepatitis B (CHB) patients.
(A) Genotype/sub-genotype distribution in AHB patients (left), AHB monoinfected patients (center), and acute HBV/HIV co-infected patients (right). **(B)** Genotype/sub-genotype distribution in CHB patients (left), CHB monoinfected patients (center), and chronic HBV/HIV co-infected patients (right). References: A1, light blue; A2, blue; A3, lavender; B, fuchsia; C: yellow; D: orange; F1b: red; F4: light green; F6: green; $^{*}p < 0.05$; $^{**}p < 0.001$.

A significant association was found between the degree of recorded liver fibrosis and inflammatory activity in liver tissue ($p < 0.001$), elevated liver enzymes ($p < 0.001$), advanced age ($p < 0.05$), and HBV viral load ($p < 0.05$). However, no significant association was found between liver fibrosis and gender ($p = 0.56$), viral genotype ($p = 0.20$), treatment ($p = 0.20$) or serological status against HIV ($p = 0.68$) (Supplementary Table 3).

3.7. Detection of mutations

3.7.1. Mutations modulating HBeAg expression

The frequency of mutations in the ORF-pC/C region found was 35.5% (77/217): in AHB patients, the frequency was 20.3% (16/79), while in CHB individuals, it was 44.2% (61/138).

Table 2 shows the frequency of mutations in this genomic region and the infecting genotype in the studied groups.

Within AHB patients, 10.1% (8/79) had the double core mutation A1762T/G1764A, 3.8% (3/79) presented the core mutation T1753C and 7.6% (6/79) had the precore mutation G1896A, mainly observed in HBV-mono-infected patients (Figure 4). These mutations were only found in gts F and C (Table 2). The double mutation A1762T/G1764A and the

TABLE 2 Frequency of mutations in the open reading frame (ORF)-pC/C region in patients with acute and chronic hepatitis B virus (HBV) mono and co-infection according to the infecting genotype.

	Acute hepatitis B (AHB)				Chronic hepatitis B (CHB)							
	HBV			HBV/HIV	HBV						HBV/HIV	
Genotype/Sub-genotype	C ($n = 2$)	F1b ($n = 51$)	F4 ($n = 5$)	F1b ($n = 8$)	A2 ($n = 13$)	C ($n = 2$)	D ($n = 12$)	F1b ($n = 28$)	F4 ($n = 19$)	A2 ($n = 20$)	F1b ($n = 26$)	F4 ($n = 6$)
T1753C n (%)	1 (50.0)	—	2 (40.0)	—	1 (7.7)	—	3 (25.0)	2 (11.1)	2 (10.5)	1 (5.0)[a]	1 (3.8)[b]	3 (50.0)[a,b]
A1762T/G1764A n (%)	1 (50.0)	5 (9.8)	—	2 (25.0)	5 (38.5)	2 (100.0)	2 (16.7)	9 (32.1)	3 (15.8)	2 (10.0)	3 (11.5)	—
G1896A n (%)	—	5 (9.8)	—	1 (12.5)	—	1 (50.0)	11 (91.7)[c]	7 (25.0)[c,d]	15 (78.9)[d]	—	—	1 (16.7)

[a]A2 vs. F4 $p < 0.05$.
[b]F1b vs. F4 $p < 0.001$.
[c]D vs. F1b $p < 0.05$.
[d]F4 vs. F1b $p < 0.05$.

mutation G1896A were mainly present in sgt F1b, in both HBV-mono-infected and HBV/HIV co-infected AHB patients (Table 2). Besides, the 50% of the individuals with AHB with these mutations were negative for HBeAg with presence of anti-HBe.

For the group of CHB patients, 25.4% (35/138) had the precore G1896A mutation, 18.8% (26/138) had the double core mutation, and 9.4% (13/138) presented the core mutation T1753C. Eight subjects had the double mutation A1762T/G1764A and the precore G1896A mutation simultaneously (Figure 4). As in AHB patients, mutations were mainly present in HBV-mono-infected patients (Table 2). In CHB mono-infected patients, the mutation T1753C was more prevalent in gt D (25.0%) (Table 2), although no significant differences were observed compared to the other gts. The mutation G1896A was found in a significantly higher proportion in gtD and sgt F4 (91.7 and 78.9%, respectively) compared to sgt F1b (25.0%) (Table 2). In HBV/HIV co-infected patients, the frequency for the mutation T1753C reached 50% for sgt F4, significantly higher than the frequency obtained for sgts A2 (5%) and F1b (3.8%) (Table 2). In this group of patients, the mutation G1896A was found only in one subject infected with sgt F4. For all the CHB individuals, the double mutation A1762T/G1764A was found in more than the 10% of the patients, and this frequency was even higher for gt C (40.0%–2/5), sgt F1b (22.2%–12/54), sgt A2 (21.2%–7/33), gt D (16.7%–2/12) and sgt F4 (12.0–3/25%) (Table 2). All samples with G1896A mutation and the 85% of the samples with the double mutation A1762T/G1764A were HBeAg (–), with presence of anti-HBe, respectively.

3.7.2. Mutations related to vaccine escape and diagnostic failure

Overall, ORF-S mutations were detected in the 7.8% of the samples (17/217). Single mutations were observed in 16 cases and double mutations in 1 case. Mutants associated with diagnostic failure and vaccine escape were detected in both, AHB (3.8%) and CHB (10.1%) patients (Figure 4). HBV gt D showed the highest mutation frequency (38.5%), followed by gts C (14.3%), A (8.5%), and F (4.7%) [(gt D vs. gt A, $p < 0.05$) (gt D vs. gt F, $p < 0.001$)]. Mutations were observed in 8 out of 12 aa residues analyzed. The most common mutated residues were P120Q/S (2.8%), D144A (1.4%), T118A, M133T/I, F134L (0.9% each), and Q129R, G130R, and S143L (0.5% each), while 4 positions (I/T126, K141, P142, and G145) did not change. Among the most frequent mutations, P120Q/S mutation was mainly present in gt F, T118A and D144A were exclusively present in gt D and sgt A2, respectively (Table 3).

3.7.3. Antiviral drug-resistance mutations analysis

Amino acid substitutions associated with resistance to antiviral therapy were detected in ORF-P in low frequency (3.2%, 7/217). The most common mutated residues were L180M (2.3%, 5/217), M204V (1.8%, 4/217), V173L (0.9%, 2/217), and A181T (0.5%, 1/217), while seven positions (rtL80, rtI169, rtS184, rtA194, rtS202, rtN236T and rtM250V) did not change (Figure 4). The rtL180M mutation, a possible cause of resistance to LMV and LdT, was detected in one AHB patient (Figure 4). In CHB patients with HBV/HIV co-infection, the following combinations of mutations were detected: rtV173L + rtL180M + rtM204V (reported to cause resistance to LMV, LdT and ETV) and rtL180M + rtM204V (reported to cause resistance to LMV and LdT) and the mutation rtA181T, a cause of resistance to ADV. The mutation V173L was registered in one CHB HBV mono-infected patient (Figure 4). Mutations related to antiviral drug-resistance were only found in patients infected with sgts A2 and F4.

3.8. Hepatitis D virus

Four patients were reactive for anti-HDV antibody detection (5.2%, 4/77) and HDV RNA was detected in one sample from a CHB patient with reactive serology. The phylogenetic analysis showed that the sequence obtained belonged to HDV gt 1 (Figure 5), in co-infection with HBV gt D. In the remaining 3 samples IgG anti-HDV + /HDV RNA-, (1 patient with AHB and 2 with CHB), the infecting HBV genotype was F.

4. Discussion

In Latin America molecular epidemiology and genomic studies of HBV are restricted to some countries. HBV genotypes A, D, and F are the most frequently detected in South America, but other genotypes have also been observed (B, C, E, G, and H) (33, 34).

The first studies performed in Argentina showed high circulation of genotype F, particularly in northern regions, where there is a large number of people of Amerindian ethnic origin. On the other hand, in large cities, such as Buenos Aires, which are cosmopolitan regions, with a large movement of local people and persons from other countries (immigration and tourism), the genotypes described have been varied, finding similar proportions of gts A, F, and D (18). In addition, in places with particular immigration patterns, such as in Misiones province, with high immigration from Eastern Europe, a preponderance of gt D was recorded (20). In recent years, the gt F has been the most frequently reported in all the provinces, mainly represented by sgts F1b and F4, followed by genotype A, mainly sgt A2 (19–26). Besides, a new variant of gt F emerged, identified as a new F sub-genotype, proposed as sgt F6 (27), showing the importance of continuous genomic surveillance and the study of its biological and clinical implications.

The present study provides information about the molecular profile of HBV and the epidemiological characteristics of

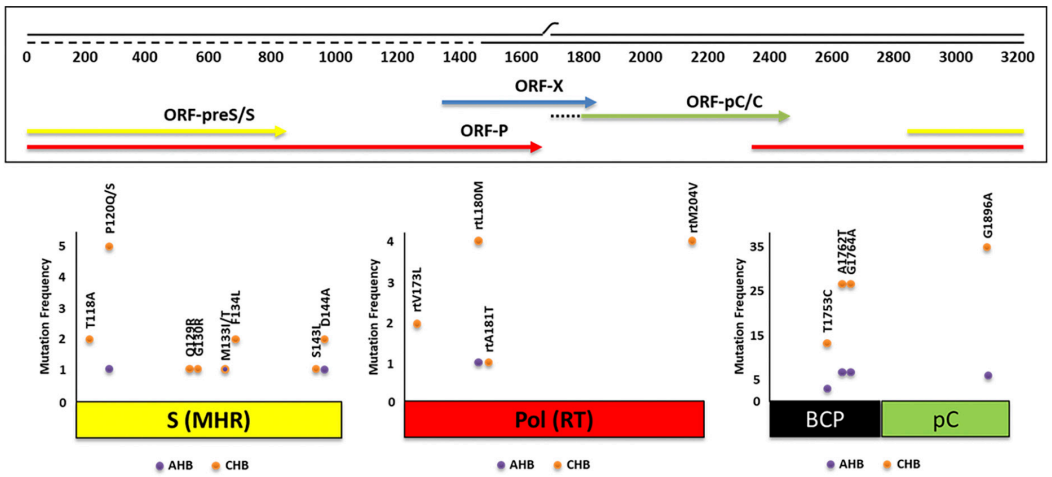

FIGURE 4
Absolute frequency of mutations obtained for three genomic regions of hepatitis B virus (HBV) [open reading frame (ORF)-S, ORF-P, ORF-pC/C] of 217 patients with acute (AHB) and chronic (CHB) infection. Some of the mutations were found simultaneously.

TABLE 3 Frequency of mutations in the open reading frame (ORF)-S region in patients with acute and chronic hepatitis B virus (HBV) mono and co-infection according to the infecting genotype.

	Acute hepatitis B (AHB)		Chronic hepatitis B (CHB)							
	HBV	HBV/HIV	HBV					HBV/HIV		
Genotype/Sub-genotype	F1b (*n* = 51)	A2 (*n* = 2)	A2 (*n* = 13)	C (*n* = 2)	D (*n* = 12)	F4 (*n* = 19)	F6 (*n* = 5)	A2 (*n* = 20)	D (*n* = 1)	F4 (*n* = 6)
T118A *n* (%)	—	—	—	—	2 (16.7)	—	—	—	—	—
P120Q/S *n* (%)	1 (2.0)	—	—	—	—	3 (15.8)	—	—	1 (100.0)	1 (16.7)
Q129R *n* (%)	—	—	—	—	1 (8.3)	—	—	—	—	—
G130R *n* (%)	—	—	—	—	—	1 (5.3)	—	—	—	—
M133I/T *n* (%)	—	1 (50.0)	—	1 (50.0)	—	—	—	—	—	—
F134L *n* (%)	—	—	—	1 (50.0)	—	—	1 (20.0)	—	—	—
S143L *n* (%)	—	—	—	—	—	—	—	—	1 (100.0)	—
D144A *n* (%)	—	1 (50.0)	1 (7.7)	—	—	—	—	1 (5.0)	—	—

patients with acute and chronic hepatitis, treated in the public health system of the province of Córdoba, Argentina, over a period of 8 years. We add updated evidence of the HBV circulating genotypes and sub-genotypes. We determined that genotype F was the most prevalent (68.7%), followed by genotype A (21.6%), in all groups studied: patients with AHB and CHB, mono-infected and co-infected HBV/HIV. A greater diversity of genotypes was found in the group of patients with CHB, compared to AHB. These results agree with most of the countries of South America, in which genotype F is the most prevalent found, since it is autochthonous from the continent (33).

The circulation profile of genotype F among acute infections presented values similar to those found historically in the northern provinces of our country and more recently in Buenos Aires (>80%) (26, 35), and higher than what was previously found in Córdoba (~50%) (19). Similarly, genotype F was the most frequent in patients with CHB, and its proportion also increased in our region (60.9 vs. 46.7% found in a previous study) (19). The high circulation of the F genotype, particularly the sub-genotype F1b, in our region would then be related to its autochthonous origin and would be the consequence of a late reintroduction from areas enriched with the F genotype (northern provinces and neighboring countries) that took place from the late 1940s to the present (35). Our region is influenced by a constant flow of students or people seeking better working conditions from northern Argentina and from neighboring countries such as Bolivia, Paraguay and Peru, which also leads to the introduction of new viral variants (33, 35, 36).

Sub-genotype F4 was mainly detected in CHB. This sub-genotype has been widely detected in Paraguay, in a frequency of 80%, and is indigenous from South America (36).

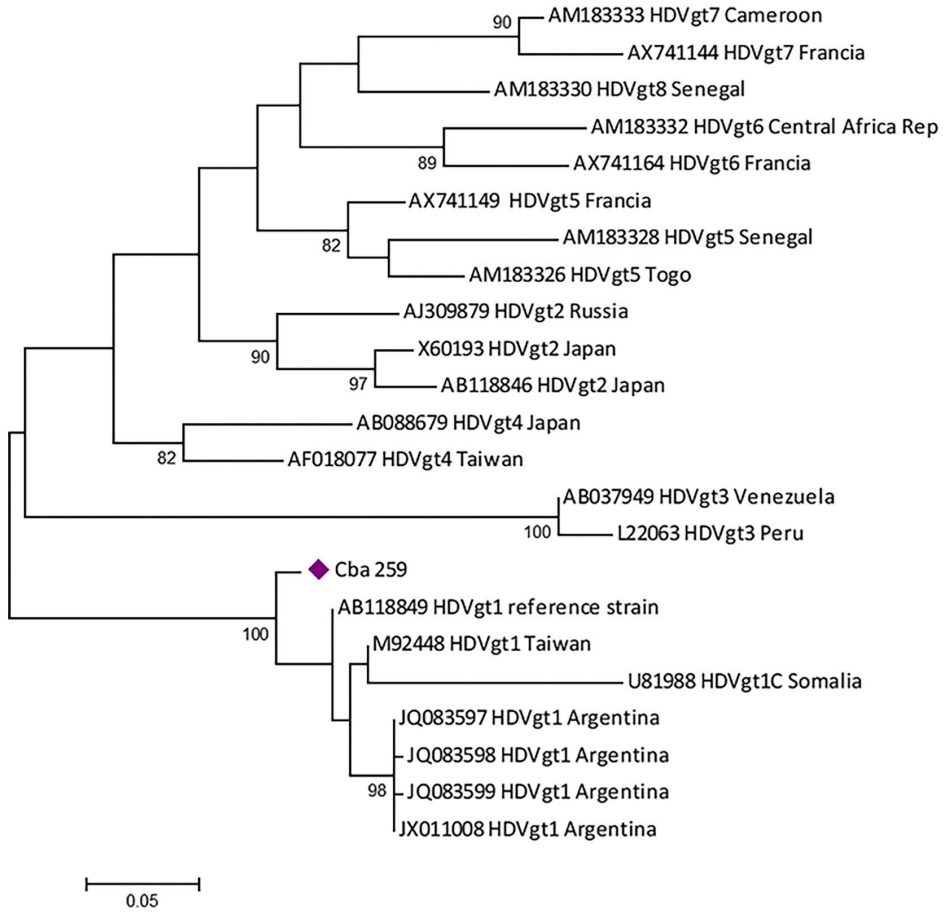

FIGURE 5
Maximum likelihood phylogenetic tree obtained using sequences of 326 nucleotides, corresponding to the partial HDAg region of hepatitis D virus (HDV), constructed with MEGA v.6 (bootstrap: 1000 replicates). The tree includes the positive sample obtained (violet diamond) and the reference sequences from each genotype available at GenBank.

The recently described sub-genotype F6 is, at the moment, exclusive to Argentina (27), and was found in our study only in CHB mono-infected patients, as previously described (19, 27).

The second genotype most frequently found in our study cohort, both in AHB and CHB patients, was A, represented mostly by sub-genotype A2. This agrees with previous studies in our country (19, 25). This sub-genotype has been associated to HBV/HIV co-infection, as demonstrated in previous reports (19, 37, 38). Accordingly, in this study the frequency of sub-genotype A2 was higher in HBV/HIV co-infected patients than in mono-infected patients. The precise reason why the sub-genotype A2 is more prevalent in HBV/HIV co-infected patients is unclear. This could be due to risky sexual behaviors in closed or semi-closed social groups. Besides, a shift in the profile of genotypes detected in co-infected patients was observed. Previous studies reported genotype A as the most frequently found in Central Argentina in this group, followed by F (19). Currently, this distribution is inverted, finding genotype F in greater proportion, followed by A. This could be due to inherent differences in sampling among the studies (sample size, geographical distribution of samples) and/or due to a global increase in the dissemination of genotype F in our region.

As previously reported in our area, genotypes B, C and D were found in low frequencies. Genotypes B and D were only detected in patients with CHB. Genotypes B and C are usually associated with the immigration of people from Southeast Asia, and genotype D with immigration from Europe and Eastern Europe (39, 40). Although genotype D is the most frequently detected in the Southern region of Brazil (a limiting country with Argentina), this has not yielded a shift in the HBV genotype profile in our region so far. In fact, some of the patients infected with genotypes B, C, and D included in this study were immigrants from Europe and Asia. These genotypes are rarely detected or detected in low frequencies in our region, so it is inferred that they do not have characteristics of high transmissibility among our population, or have evolutionary disadvantages compared to the two preponderant genotypes F and A.

Various studies have shown the existence of dissimilar characteristics between the different viral genotypes, in relation to the clinical course, HBV viral load, and, particularly, to the HBeAg seroconversion rate (41–43). In our study we only found that individuals infected with genotype A presented a significantly higher HBV viral load than those infected with genotype D among CHB patients. No significant differences between the rest of the clinical features analyzed and the genotypes were found. No conclusions can be drawn in this regard due to the large proportion of genotype F and the low number of samples analyzed for other genotypes, added to the scarce record of clinical variables available in this study.

Hepatitis B virus evolution occurs through mutation and recombination processes (44, 45). The existence of mutations with clinical implications in the HBV genome poses a challenge for the design of diagnostic assays and treatment strategies, and is considered as a potential threat to long-term success of vaccination programs (46, 47). The analysis of nucleotide and amino acid substitutions showed that the observed changes occurred in both acutely and chronically infected patients, mono-infected (HBV) and co-infected (HBV/HIV), but with a higher prevalence in chronic mono-infected patients.

In this study, immune escape mutations were detected in 7.8% of the HBV sequences analyzed, similar to those described in other cohorts from Argentina (7.5–10.7%) (25), China (9.0%) (47), and Spain (6.6–12.5%) (48). Unlike what was found in previous analyzes (32, 49), only 2 of the 12 analyzed aa residues had a mutation frequency greater than 1% (P120Q/S, 2.8%; D144A, 1.4%) and the rest were below this value. In addition, similar to that observed in Argentine patients (25), this study found a significantly higher rate of escape mutations in genotype D (30.8%) than in genotype F (4, 7%).

The most frequently detected HBV mutation with proven vaccine escape properties is the G145R mutation (32). Although other putative escape variants included in the analysis, such as P120T/A and D144A, have been reported (32, 50), evidence for the escape role of these variants is incomplete. Despite the continuous detection of these vaccine escape variants in different parts of the world (32, 50, 51), their dissemination in the population and the consequent reduction in the efficacy of anti-HBV vaccination programs have not generated, to date, a problem that threatens public health, even in areas of high prevalence (52, 53). These mutants involved in immune evasion were found in very low frequency among AHB patients in this study, in accordance with the described by Rodrigo et al. (26), in the metropolitan area of Buenos Aires. Mutation G145R and P120T/A variant, as well as mutations involved in diagnostic failure, were not identified among genotype F (predominant in the region) in our cohort of patients, suggesting that mutations in the ORF-S are not a matter of major concern at this moment.

Mutations in the BCP/pC region associated with a higher risk of HCC, higher HBV viral loads and the decrease or absence of HBeAg (which would contribute to HBeAg seroconversion) have been described (18, 54–57). In our samples, mutations in this genomic region were mainly found in CHB patients. Mutation G1896A was found in a higher proportion in genotypes D and F4 among CHB mono-infected patients, associated to HBeAg seroconversion, in accordance with previous studies (18).

The goal of the treatment in patients with CHB is to prevent the progression of liver disease, the development of cirrhosis and HCC (58). Different studies have shown that sustained suppression of viral replication is associated with remission of liver disease (59). Prolonged treatments with nucleos(t)ide analogs induce the appearance of mutant HBV strains resistant to the different types of drugs used (60). In our study, amino acid substitutions related to antiviral resistance were rare in the AHB patients. Only one sample had the rtL180M mutation, associated to lamivudine and telbivudine resistance. This is consistent with previous studies, which reported low prevalence of mutations in naïve patients (25). In the population of patients with chronic infection, combinations of antiviral resistance mutations to lamivudine, telbivudine, adefovir and intermediate resistance to entecavir were found. However, these mutations were observed in low frequencies and only in HBV/HIV co-infected individuals under antiretroviral treatment (HAART regimens that included tenofovir) (one of them had previously been treated with lamivudine). The results are encouraging for two reasons: (1) there is low circulation of strains with antiviral resistance mutations, and (2) most of the drugs for which resistance was found are not currently the first-line treatment of choice. However, the continuous surveillance of these circulating variants is recommended, due to the possible appearance of mutations with resistance other drugs currently used.

This work reports, for the first time, the circulation of HDV in the central region of Argentina. We found a prevalence of total anti-HDV similar to the previously reported worldwide (61). The sequenced sample belonged to HDV-1, in accordance with previous studies performed in Amerindians from Misiones province (35) and in blood donors of Buenos Aires (22). However, association with HBV sub-genotype D3 was not previously reported in our country. Although HDV infection has been associated with a worse clinical outcome, in our cohort, HDV infection was observed in non-hospitalized subjects. The presence of anti-HDV antibodies in one subject with AHB could indicate a co-infection (or simultaneous infection) with HBV and HDV. More studies including a larger number of samples and the follow-up of the patients over time are needed to elucidate the impact of HDV in our area.

Some limitations in our study need to be considered: (1) we did not use next generation sequencing (NGS) techniques for sequencing samples (we only used Sanger method), so it was not possible to detect and study quasispecies; (2) the lack of full-length sequence data; (3) no data about vaccination were

collected in this study. Taking into account the mean age of our cohort and that the vaccination programs anti-HBV in Argentina started in 2000, it is very likely that the great majority of included patients were unvaccinated; in this sense, reinforcing vaccination campaigns in adults is a priority; (4) complete clinical (including markers of liver disease) and therapeutic data were not available for all samples studied. Particularly in the case of positive HDV samples, there were no liver histological data or the clinical evolution of the patients; and (5) the potential lack of generalizability to other regions in South America.

In conclusion, we detected a slight increase in the circulation of genotype F our region, particularly sub-genotype F1b. The high frequency of detection of this genotype among AHB patients suggests transmission advantages over the other genotypes. A low rate of mutations was detected, especially those with antiviral resistance implications, which is an encouraging result. The evidence of HDV circulation in our region (reported for the first time) alerts the health system for its search and diagnosis. These results give scientific evidence of the HBV and HDV circulation in South America, providing valuable information that could be used for health effectors, as well as for the design of treatment guidelines (with particular interest in HBV genotype F). This becomes relevant in the framework of the Strategy for the elimination of viral hepatitis proposed by the WHO for 2030 (62), which urges countries to promote knowledge to guide responses, especially in countries where these infections are poorly studied.

Data availability statement

The data presented in this study are deposited in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/), accession numbers OM333932 to OM334148, OM456810 to OM456985, and ON751779.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethics Committee for Child and Adult Health Research of the Ministry of Health of Córdoba Province, Argentina (RePIS N° 2701). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

GC, MP, and VR conceived and designed the experiments. GC, MS, PS, MR, and MP performed the experiments. GC, MP, VR, and AC analyzed the data. CM, RC, JD, and MB contributed reagents, materials, and analysis tools. GC, JD, MP, and VR wrote the manuscript. RC carried out the clinical characterization of the patients. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1057194/full#supplementary-material

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Prevalence of HDV infection in people living with HIV: Data from a multicenter Italian cohort

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Objectives: The development of novel antiviral agents active against Hepatitis Delta Virus (HDV) might change the natural history of chronic infection, reducing the risk for end-stage liver disease. People living with HIV (PWH) are at risk for bloodborne pathogens infection, but limited data on epidemiology of HDV infection is available in this setting. The aim of this study was to investigate HDV prevalence and attitude toward HDV testing and treatment in infectious diseases centers.

Methods: A cross sectional survey was performed among centers participating in the CISAI (Coordinamento Italiano per lo Studio dell'Allergia in Infezione da HIV) Group. The survey addressed anti-HDV prevalence and HDV-RNA detectability rates in PWH as well as perceived obstacles to treatment.

Results: Overall, responses from ten sites were collected. Among participating centers, 316 PWH with HBV chronic infection are currently followed. Of them, 15.2% had positive anti-HDV antibodies, while 13.9% were not tested yet. Overall, 17% of anti-HDV positive PWH tested at least once for HDV-RNA had active HDV infection, and 71% of them had advanced liver disease. Most infectious diseases centers intend to treat locally HDV infection with upcoming anti-HDV drugs, but some concerns exist regarding treatment schedule.

Discussion: HDV testing needs to be implemented in PWH. At present, few patients followed in the CISAI centers seem to be candidate to receive new direct active anti-HDV agents, but repeated HDV-RNA measures could change this proportion.

KEYWORDS

HIV, bulevirtide, treatment, HDV, prevalence

Background

According to a recent meta-analysis, approximately 12 million people worldwide live with hepatitis Delta virus (HDV) infection, and up to 64% of anti-HDV positive people have chronic HDV replication (HDV-RNA) (1). HDV is a defective virus that requires hepatitis B virus (HBV) surface antigen (HBsAg) to cause liver infection and disease. Chronic HDV infection poses patients at risk for liver cirrhosis, clinical decompensation, and development of hepatocellular carcinoma (HCC) (2). Thus, HDV infection is a major health problem that needs to be addressed in order to reduce liver-related mortality.

The risk of developing HBV and HDV infection is higher in people living with HIV (PWH), and PWH are at higher risk of developing chronic HBV infection (3, 4). Moreover, PWH coinfected with HBV experience more frequently cirrhosis and its complication than people living without HIV (5). Additionally, data from the Swiss HIV cohort study highlight that HDV infection is strongly associated with overall and liver related death as well as with the occurrence of HCC in PWH (4). Reasons for the exceeding risk are still unclear, but it has been supposed that impaired immune surveillance due to HIV infection could promote the development of HCC (6).

Notably, HDV prevalence has changed over time, and it is difficult to understand the current extent of the problem. In Italy, the proportions of anti-HDV positivity in PWH dropped from 28% in 1997 to 4% in 2011, then it rebounded to 8% in the period 2012–2015 (7).

Persistent HDV replication is the only identified predictor of liver-related events, including cirrhosis and HCC, in anti-HDV positive people. Additionally, levels of HDV-RNA seem to predict liver disease progression (8, 9), while persistent HDV-RNA suppression following treatment results in reduced liver-related mortality and increased cumulative event free survival (10, 11). Although the clinical significance of HDV-RNA is clear, data on HDV prevalence usually focuses on anti-HDV seroprevalence, while a few studies reported on HDV viraemic infection.

The aim of this survey was to investigate the prevalence of anti-HDV and replicative HDV infection as well as the attitude toward HDV treatment in PWH, in a large Italian HIV network.

Methods

A cross sectional survey was performed among centers participating in the CISAI (Coordinamento Italiano per lo Studio dell'Allergia in Infezione da HIV) Group, a collaborative group of Italian HIV clinics (12). The survey included 10 questions and was advertised by email to the HIV clinic directors. Following first advise, a remind was sent a few weeks later. Participation was voluntary and not compensated. Participating sites provided raw data that were subsequently elaborated. Although participation was not anonymous, no information on characteristics of participating sites was asked. Results of the survey were discussed during the CISAI annual meeting in May 2022.

In the survey, the first questions addressed the prevalence of anti-HDV and HDV-RNA in PWH with detectable HBsAg, the proportion of untested PWH, and features of HDV-related liver disease (i.e., grade of liver fibrosis and presence of liver cirrhosis).

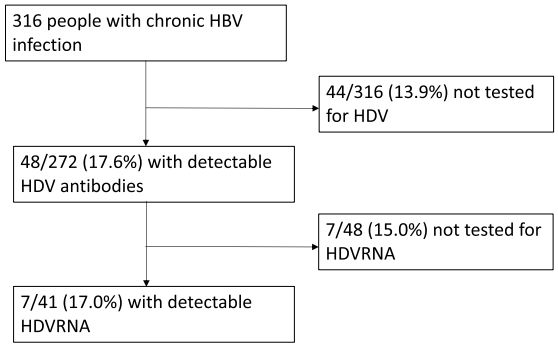

FIGURE 1
Flow chart of the study population.

According to the study coordinating center procedures, a cut-off of 14 kPa at transient elastography was used for the diagnosis of cirrhosis. Metavir F3 fibrosis was defines as liver stiffness between 10.1 and 14, while Metavir F2 for liver stiffness between 8 and 10 kPa (13).

Previous treatment with interferon was also investigated. Regarding upcoming treatment options for HDV, we asked whether eligible patients would receive treatment on-site or they would be addressed to an hepatologist referral center. Finally, a close ended question was used to investigate potential issues related to HDV treatment.

Results

Ten centers answered the questionnaire. Overall, 316 PWH with HBV chronic infection referred to the participating centers (**Figure 1**). Of them, 48 (15.2%) had detectable anti-HDV. Of the remaining patients, 44 (13.9%) were not tested for anti-HDV antibodies. A *post hoc* power analysis, performed using the OpenEpi software (14), revealed that our sample size allowed a 99% confidence level that the true anti-HDV prevalence in our cohort is between 14 and 16%.

Among anti-HDV positive patients, 15% (7/48) had never been tested for HDV-RNA. Of tested patients, 7 (17%) had detectable HDV-RNA and were thus regarded as having active HDV co-infection.

Of PWH with active HDV, liver elastography was available for 6 patients and revealed liver cirrhosis in 2 cases, who also present with laboratory and clinical signs of liver cirrhosis, Metavir F3 in 2 and Metavir F2 liver fibrosis in 2 cases. One additional patient received liver transplantation for HCC; after transplant, he did not experience HBV reactivation so far. All data on liver fibrosis were collected according to liver stiffness at transient elastography and were referred to March 2022, except for one patient who received liver elastography in June 2022 as she was diagnosed with HBV/HDV infection at the beginning of the SARS-CoV-2 pandemic. In the meanwhile, she experienced oropharyngeal carcinoma requiring combined surgery, chemotherapy and radiotherapy. Thus, elastography was postponed in order to limit the number of hospital accesses; aspartate aminotransferase (AST) to Platelet Ratio Index (APRI) and Fibrosis-4 (FIB-4) indexes ruled out liver cirrhosis. Overall, 3 (42.9%) patients with active HDV had previously been treated with interferon without HDV eradication.

Regarding the attitude toward upcoming treatment options, all but one centers responded that active HDV co-infection would be treated on-site once new drugs become available. The last center answered that they usually address their patients with hepatic issues to a gastroenterology referral center for chronic liver hepatitis. Reported potential barriers to HDV treatment initiation were deemed: the need to increase frequency of medical visits and blood tests (n = 1); the subcutaneous route of administration (n = 2), the unclear length of treatment schedule (n = 1), and the potential risk for drug-to-drug interactions (DDI) (n = 1).

Discussion

In the present study, we report a seroprevalence rate of HDV infection among PWH HBsAg carriers of 15.2%, while the 13.9% is still waiting for anti-HDV testing. In countries without a generalized HIV epidemic, an epidemiological association between HDV and HIV infection has been reported, probably related to the shared transmission routes (1). HDV prevalence rate we found was consistent with estimated prevalence in HBsAg-positive populations from hepatology clinic in Europe (1), but it was slightly different from those reported by the Swiss and Italian cohorts up to 2015 (4, 8, 15). Indeed, HDV prevalence was 18% in HBsAg positive PWH enrolled between 1988 and 2014 in the Swiss HIV cohort study (4). The ICONA foundation reported an 8% HDV seroprevalence rate in the period 2012–2015 (7). By contrast, a multicentre study in Northern Italy found that approximately one third of PWH seen at one of the participating centers in 2010 had positive HDV serology (13). Of note, our data may partially overlap those from the ICONA and from the Northern Italy, as some CISAI centers also participate in these cohorts. However, our data are updated to 2022 and thus provide a picture of the current epidemiology of HDV infection in PWH.

The proportion of patients untested for anti-HDV is lower than previously reported in the Italian cohorts (7, 16). This data could reflect an increasing attitude toward anti-HDV testing in clinical practice, that might be related to the upcoming availability of anti-HDV drugs. Indeed, up to 2020, no HDV direct-acting antiviral agent was available. Pegylated Interferon was the only drug approved by the European Medicines Agency (EMA), although it did not receive the Food and Drug Administration approval (16). Unfortunately, treatment with pegylated interferon was limited by low efficacy rates, high risk for adverse events and possibility of late relapse of HDV infection (17–19). Recently, new molecules targeting host factors have been developed (20). Among them, bulevirtide is an entry-inhibitor that received conditional marketing authorization by EMA in 2020, based on two small phase II studies (16). Clinical trials and real-word experiences showed that bulevirtide reduced HDV-RNA and normalized alanine aminotransferase levels. However, in clinical trials the treatment duration was limited to 24 weeks and off-treatment virological response was not reported (21, 22). Additionally, clinical trials focused on viraemic individuals and used the combination of > 2log decline in viral load and normalization of alanine aminotransferase, in spite of virological suppression, as surrogate marker for efficacy (21, 22). Further efforts are needed to ensure that all PWH with ongoing HBV infection receive HDV screening, according to international guidelines.

The rate of replicative HDV infection we found was as low as 17%. According to the literature, HDV-RNA detectability rate widely vary (4, 23, 24). Notably, our survey did not focus on viral nuclear extraction protocols and type of assay used to assess presence of HDV-RNA. Additionally, we did not evaluate whether PWH received single or repeated testing for HDV-RNA. Thus, we could not argue on the possibility of false negative results.

The performance of different techniques for the assessment of liver fibrosis in HDV infected patients has not been well-studied so far. While liver biopsy is historically considered the gold standard for disease staging, several non-invasive fibrosis tests have shown to be accurate in evaluating the presence of significant fibrosis and liver cirrhosis in HBV and HCV chronic infection (25). However, validation of these tests in the setting of chronic HDV is still pending. Novel tests recently studied in the setting of HDV are the Delta Fibrosis Score and the D4FS, that have shown promising results in the assessment of advanced liver fibrosis and cirrhosis, respectively (25). Although ideal cut-offs of liver stiffness for staging of liver fibrosis in HDV infected patient with transient elastography are not available, we asked centers to report on liver fibrosis according to liver stiffness, as it is largely used in the setting of HBV infection and easy to report. However, the possibility that liver disease staging with these cut-offs could not be accurate should be acknowledged.

Both hepatologists and infectious diseases specialists usually manage patients with chronic viral hepatitis. However, depending on local and regional organization, patients may be sent to referral centers. As we aimed at investigating HDV in PWH, we conducted the survey among infectious diseases specialists. Notably, among participating centers only one reported that individuals eligible to upcoming HDV treatment would not be treated locally. Concerns regarding treatment included the route of administration and uncertainty regarding treatment duration as well as the need to increase controls despite the recent SARS-CoV-2 pandemics. At present, data on the optimal treatment duration and post-treatment efficacy using bulevirtide are pending (26, 27). Results from phase 3 clinical trials should be soon available and might be helpful in order to address these concerns. Regarding potential DDIs, bulevirtide is a CYP3A4 inhibitor, thus it likely presents some risk. However, as limited information are available, further studies are needed (28).

In summary, despite 15.2% anti-HDV prevalence, we found that 17% of anti-HDV positive PWH harbored active HDV infection and were thus eligible for treatment with bulevirtide and/or other anti-HDV drugs currently under development. HDV chronic infection is difficult to treat, and no standardized screening strategies have been implemented in Central Europe so far (29). Given that new drugs are on the horizon, implementing screening strategies for HDV infection and HDV-RNA testing is pivotal, especially in populations at high risk for HDV infection.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Local Ethical Committees participating in the CISAI cohort. Indeed, CISAI supports a prospective, observational, multi-center study created to assess the incidence of adverse events

in patients receiving new antiretroviral drugs in clinical practice. It is an online pharmacovigilance program involving 22 Italian Infectious Disease Departments. The coordinating center is ASST Fatebenefratelli Sacco-Milan, Italy. The Project has an Internet site (http://www.cisai.info). The survey of the present study was addressed to the site directors of the participating centers. Patients evaluated in the survey were already enrolled in the project. Participation in the observational multi-center study requires a signed informed consent. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LN designed the study and wrote the manuscript. AD, BM, and PB supervised the findings of this work. ER performed the computations and worked out the technical details. EP, GC, and GO worked out the clinical aspects of patients with HDV infection. AC, GP, LV, CM, and FV contributed to the interpretation of the results. All authors discussed the results, contributed to the final manuscript, and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Low prevalence of hepatitis delta infection in Cuban HBsAg carriers: Prospect for elimination

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Introduction: Infection with hepatitis delta virus (HDV) is one of the most severe hepatitis B virus (HBV) complications, with a more rapid progression to cirrhosis and an increased risk of hepatic decompensation and death. Data on HDV infection in Cuba are limited. The aims of our study were to determine the HDV prevalence in HBsAg carriers and to characterize the HDV strains circulating. The data were used to assess the possibility of HDV elimination in the Cuban HBV epidemiological setting.

Methods: Five hundred and two serum samples from the same number of HBsAg carriers collected in the period 2006–2019 from all over the country were tested for anti-HDV total antibodies. If positive, the samples were analyzed for HDV-RNA using Real-Time RT-PCR targeting the ribozyme and HD antigen domains followed by genotyping based on phylogenetic analysis.

Results: Two samples were anti-HDV positive [0.39% (95% CI 0.11–1.44)]. One of them was also HDV-RNA positive. Clinically, the patient with active HDV infection had compensated liver cirrhosis. Phylogenetic analysis showed that the virus belonged to genotype 1 and thus clustered with contemporary strains from North America, Europe, Middle East, and Asia.

Discussion: This is the first HDV study, including molecular detection and virus characterization, done after the introduction of the universal childhood anti-hepatitis B vaccination. The very low prevalence of HDV infection in HBsAg carriers combined with the high HBV vaccination coverage of all newborn children, of previously identified risk groups, and of the general population currently under 40 years of age suggests that HDV elimination is feasible in Cuba if the success in HBV control is maintained.

KEYWORDS

hepatitis delta virus (HDV), hepatitis B virus, HBsAg carriers, hepatitis B vaccination, HDV elimination

Introduction

Hepatitis delta virus (HDV) is a unique RNA virus requiring hepatitis B virus (HBV) for replication and infection of hepatocytes (1). Recent data estimated that more than 10% of people with chronic HBV infection are co-infected with HDV, yielding a global prevalence of 0.80% in the general population, and resulting in a total of 48–60 million persons presumably infected with HDV worldwide (2, 3).

The prevalence of HDV is usually assessed based on HDV antibody positivity among HBsAg carriers. Implementation of routine hepatitis B vaccination of children and other population groups in industrialized countries resulted in a considerable decrease of HDV prevalence (4). The impact of the disease in low-income countries is largely unknown due to a lack of awareness and of adequate diagnostic tools. In addition, available treatments are often suboptimal.

Hepatitis delta virus belongs to the genus *Deltavirus* and was recently reclassified into a new family Kolmioviridae and a new realm called *Ribozyviria* (5). HDV has a genome size of 1.7 kilobases (kb) and phylogenetic analyses have distinguished eight genotypes 1–8, which differ by up to 30% in their RNA sequence. Genotype 1 is present worldwide, while genotypes 2–8 were found in more specific geographical areas: HDV-2 and –4 are of Asian origin. HDV-3 is found in South America and HDV-5 to 8 were reported mainly from Africa and more recently in Brazil (1, 6–8).

Hepatitis delta virus genotype 1 has been associated with a broad spectrum of pathogenicity, while HDV genotype 2 is normally linked to milder forms of liver disease. HDV-3 has been reported in connection with a severe form of fulminant hepatitis, while HDV-4 is often related to mild liver disease although a variant of genotype 4 seems to increase the risk for progression to chronic hepatitis and cirrhosis. The more recently identified genotypes 5–8 from Africa are less well characterized (6–8).

Cuba is an HBV low prevalence country with a predominance of subgenotype A2 and HBsAg serotype adw2 (9). Three doses of hepatitis B vaccine are recommended at 2, 4, and 6 months of age and 1 dose for newborns within 24 h of birth. The high vaccination coverage of more than 95% in the study period has led to HBV control, with an incidence rate of 0.5/100.000 population in 2020 (10). In line with the WHO global strategy of hepatitis elimination by 2030, Cuba has set up a National Strategic Plan for the Prevention and Control of Sexually Transmitted Diseases including viral hepatitis (11). However, data regarding HDV infection is limited with only one serological study done 32 years ago, when the epidemiological context of HBV infection was very different from today (12). Therefore, the main aims of our study were to determine the HDV prevalence in Cuban HBsAg carriers and to characterize the HDV strains present in the country. The data were used to assess the possibility of HDV elimination in the Cuban HBV epidemiological setting. Additionally, the study provides reliable information for worldwide HDV prevalence estimates to guide international HBV and HDV control programs toward global elimination.

Materials and methods

Study population

Five hundred and two serum samples received at the National Reference Laboratory of Viral Hepatitis at the Institute of Tropical Medicine Pedro Kouri (IPK) to confirm HBsAg positivity and/or to perform HBV molecular diagnosis between 2006 and 2019 from all over the country were included in this study. The sera had been stored at −20°C and sample information was retrieved from laboratory books or dedicated databases. Two samples had been collected in 2006, 2 in 2007, 9 in 2008, 6 in 2009, 1 in 2011, 37 in 2014, 100 in 2015, 92 in 2016, 143 in 2017, 86 in 2018, and 24 in 2019. Two hundred and ninety-one samples were received from hospitals in Havana (Tertiary Care), 76 sera were from the Eastern, 31 from the Central and 75 from the Western part of Cuba (**Figure 1**). Twenty-nine samples lacked data about the geographical origin of the patients. In the context of our study, the doctor in charge of a patient with detectable HDV-RNA was informed and a new serum sample for virological follow-up was requested.

Some demographic, epidemiological, clinical, and virological characteristics of the study population are shown in **Table 1**.

Ethics approval and consent to participate

The study was conducted in compliance with the Declaration of Helsinki and using Good Laboratory Practices. The specimens tested for this research were residual samples received for HBV serological and/or molecular analysis. The research was approved by the Ethics Committee of the Institute for Tropical Medicine in Havana, Cuba (CEI-IPK 05-16). In case of positive results, the doctor in charge was informed. Written informed consent was obtained from the patient with active HDV infection (HDV-RNA positive) for reviewing the clinical history and for taking serum samples for the follow-up of the HBV and HDV infection status.

Laboratory investigations

Detection and confirmation of the HBsAg was done with reagents and technology from Tecnosuma Internacional S.A. (UMELISA HBsAg PLUS and UMELISA HBsAg

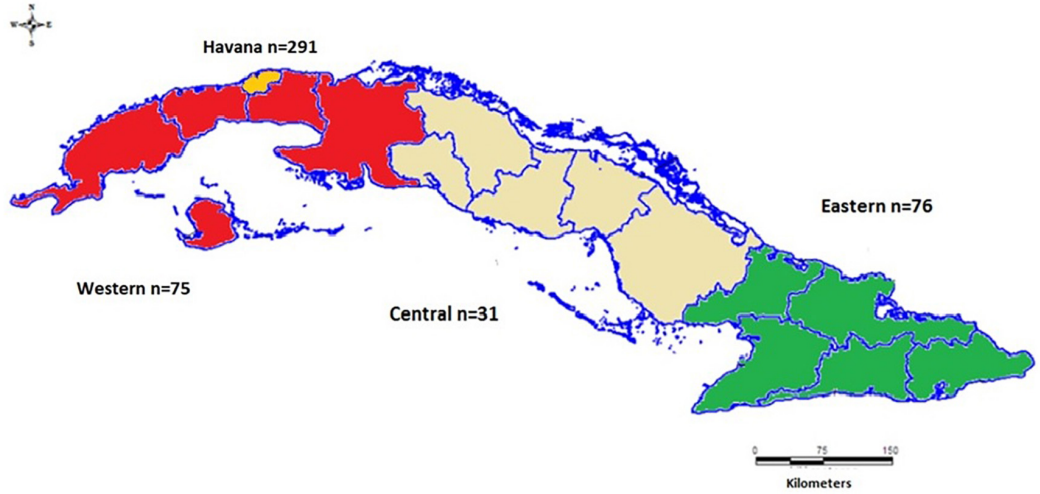

FIGURE 1
Map showing the main regions of Cuba and Havana as well as the number of samples originating from there.

Confirmatory test). Total antibodies against HDV were detected with commercial enzyme linked immunosorbent assays (Dia.Pro, Italy). Both assays were performed according to the manufacturer's instructions.

Viral RNA was extracted from 140 μL of serum using the QIAamp Viral RNA mini kit (Qiagen GmbH, Hilden, Germany). Five μL of RNA were denatured with 45 ng random primers and 10 nmol nucleotides for 5 min at 72°C. Reverse transcription (RT) was then performed for 80 min at 50°C using 200 U SuperScript III reverse transcriptase and 40 U RNaseOUT recombinant RNase inhibitor (Invitrogen, Karlsruhe, Germany) (13). RT-PCR for HDV detection was done as described previously (14). Five μL of cDNA were added to TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, United States) as well as 0.5 μM primers and 2.5 μ M probe.

Fragments for HDV genotyping were amplified using primers 480as, 710s, 1302das, rv900, fw900_2 and 320ds (13) as well as 1170s (5′-ctcgtcttchhcggtcaacctc-3′, Andernach et al., unpublished). cDNA synthetized as previously described was used with the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, United States). The PCR was done using 0.8 μM of primers, 0.5 mM of MgCl2 and 0.02 U/μl of the polymerase and the following amplification conditions: 98°C for 30 s, 40× (98°C for 10 s, 54°C for 30 s, 72°C for 60 s) and a final elongation at 72°C for 7 min. In addition, RNA was used with the One step RT-PCR kit (Qiagen GmbH, Hilden, Germany) and 0.5 μM of primers, 1.5 mM of MgCl2 and 1 μl of enzyme mixture per reaction. The cycling conditions were: 50°C for 30 min, 95°C for 15 min, 40× (95°C for 30 s, 54°C for 30 s, 72°C for 60 s) and a final elongation step at 72°C for 10 min.

PCR products were purified using the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and sequenced using the ABI Prism Big Dye Terminator cycle sequencing reaction kit (Applied Biosystems, Foster City, CA, United States) in an ABI3130xl genetic analyzer.

Sequences were edited with SeqScape v2.5 software (Applied Biosystems) and BioEdit Sequence Alignment Editor (version 7.0.9.0, Ibis Biosciences, Carlsbad, CA, United States) and aligned with reference sequences of the 8 HDV genotypes and with similar sequences obtained by BLAST.[1] Phylogenetic analyses were conducted with MEGA version 6[2] and phylogenetic trees were constructed using the neighbor-joining method and the Kimura 2-parameter model. The bootstrap method with 1,000 replications was used as measure of the robustness of each node.

Sequences obtained during this study were submitted to the GenBank Nucleotide Sequence Database under accession number: [MW273290].

Statistical analyses

A Microsoft Excel 2010 database was created for data analysis. The Chi-square test and the confidence interval (CI) calculations were done using GraphPad 7.0. Results were considered to be statistically significant when $p < 0.05$.

Results

Two of the 502 sera were positive for HDV antibodies (0.39%, [95% CI 0.11–1.44]). One of the positive sera had been

1 https://blast.ncbi.nlm.nih.gov
2 www.megasoftware.net

TABLE 1 Demographic, epidemiological, clinical, and virological characteristics of the 502 study participants, Cuba 2006–2019.

Parameters		*N*	%
Age	Children (0–≤18 years)	24	4.78
	Adults (>18 years), median age 42 years, and interquartile range [33–51]	188	37.45
	No data (adults)	290	57.77
Sex	Male	63	12.54
	Female	112	22.31
	No data	327	65.13
Purpose of HBV testing	Follow up of children of HBsAg + mothers	19	3.78
	Surveillance of pregnant women	91	18.12
	Surveillance of acute infection	6	1.19
	Diagnosis of severe hepatitis	7	1.39
	Follow up of chronic HBV infection	220	43.82
	Others	159	31.6
Co-infections	HBV/HCV	1	0.19
	HBV/HIV	90	17.92
	HBV/HCV/HIV	3	0.59
	None known	408	81.2
HBV-DNA (PCR or qPCR)	Detectable HBV DNA	146	29.08
	Non-detectable HBV DNA	123	24.5
	Not tested	233	46.4

collected in 2015, the other in 2017. Both positive samples were from male patients, between 50 and 60 years old and with chronic HBV infection. None of the two patients had an HCV or HIV co-infection. Also, in both patients HBeAg was negative and HBV-DNA was undetectable. HDV-RNA was detected only in one of them.

Clinically, this patient had compensated liver cirrhosis with portal hypertension with only splenomegaly and esophageal varices grade 1. Serum alanine transaminase (ALT), aspartate transaminase (AST), and gamma-glutamyl transferase (GGT) levels were increased, while the alkaline phosphatase (ALP) and total serum bilirubin (TSB) values were in the normal range. Other hematological and biochemical parameters like creatine, albumin, platelets, and International Normalized Ratio (INR) were also in the normal range. The patient had been under Lamivudine therapy during the last 5 years. After the HDV diagnosis, he received Pegylated Interferon Alpha therapy during 1 year. At the end of the treatment the HDV-RNA and the HBV-DNA were undetectable, while the HBsAg remained positive and the enzymes ALT and AST were in the normal range [median ALT before treatment 117 vs. median post treatment 49 ($p = 0.05$), AST: 89 vs. 51 ($p > 0.05$)]. The GGT level remained high as a marker of fibrosis.

Sequence data covering nucleotide positions 1–439 and 660–1,682 of the HDV genome (according to US-2 sequence, accession number L22066.1) were obtained. Phylogenetic analysis of the combined 1,462 nucleotides showed that the sequence from Cuba belonged to genotype 1 and thus clustered with contemporary strains from North America (United States), Europe (Spain, Portugal, France, Germany, and Italy), Middle East (Iran and Turkey), and Asia (China) (**Figure 2**).

Discussion

Although overlapping, the prevalence of HDV does not always coincide with that of HBV. In the past years, HBV vaccination and sexual limitations driven by the risk of AIDS have led to the control of HBV with a significant reduction of the number of HBsAg carriers in many countries. Deprived of HBV infections, the circulation of HDV has noticeably declined principally in the industrialized world (4, 15).

The anti-HDV prevalence found in the present study is very low. In the only other study done in Cuba in 1988, an anti-HDV positivity of 8.3% was detected (11). While we cannot exclude false positive results in the previous study, the ELISA kit used in the present study is expected to reliably detect HDV antibodies with reported sensitivity and specificity values of above 98% (16). The difference may be related to the success of the Cuban hepatitis B prevention and control program, since HBV vaccination is the most influential factor concerning the prevalence of both diseases (17). The vaccination strategy included in the National Immunization Program in 1992 comprised vaccination of all newborn children with the first dose provided in maternity hospitals, as well as vaccination of risk groups to prevent infection before potential exposure. In 2000, a vast vaccination campaign targeting all people less than 20 years of age was done (18). Therefore, the majority of the Cuban population currently under 40 years old is vaccinated against hepatitis B. After 28 years of nationwide vaccination, the rate of new infections has been drastically reduced (from 20.3/100.000 population in 1992 over 2.2/100.000 in 2001 to 0.5/100.000 in 2020) (10). Other measures for HBV control included the screening of all pregnant women, of blood and blood products, the surveillance of children born to HBsAg positive mothers using serological or more recently molecular techniques and education of the population. The considerable reduction of HBV cases may have influenced the HDV epidemiology and thus its currently very low prevalence rate.

Consistent global data on the prevalence of HDV are lacking because of different reasons such as lack of testing of HBsAg carriers for HDV infection or non-availability of high quality anti-HDV antibody assays and there are considerable geographical variations (3, 19, 20). Countries in Asia have

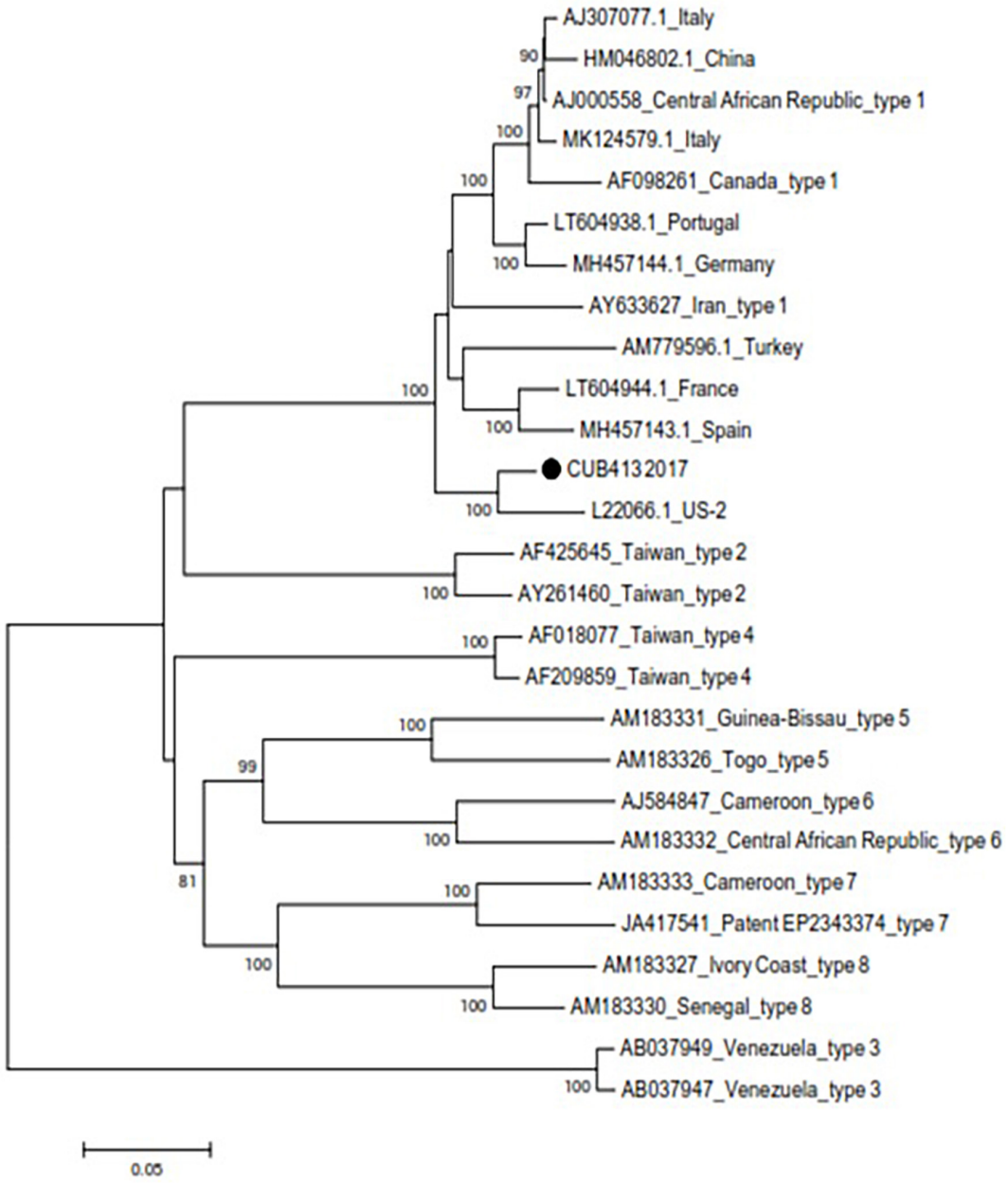

FIGURE 2
Phylogenetic tree based on the neighbor-joining method and the Kimura 2-parameter model using the partial genome of hepatitis delta virus (HDV). The sequence from Cuba is marked with a black dot. Each reference sequence is identified by GenBank accession number, country of origin, and genotype. Other genotype 1 sequences obtained by BLAST are identified by GenBank accession number and country of origin. Bootstrap values above 70 are shown at the nodes and genetic distance may be estimated based on the bar below the tree.

reported prevalence rates between 4.4 and 60% (21–23), while in sub-Saharan Africa HDV prevalence ranged from 0 to 50% in relation to the clinical picture of the patients (12). In Europe, the situation is complex because of immigration (24–27) and also countries in the Western world with low HBV prevalence, such as the United States, report diverse HDV prevalence rates (28, 29). The Western Amazonia represents one of the places with the highest prevalence of HDV in the world (30), while other Latin American and Caribbean countries have low prevalence rates (31, 32). HDV control or elimination, however, is only possible via successful HBV immunization programs (17).

The HDV RNA positive patient was born before the introduction of routine childhood HBV vaccination in Cuba. In a recent study analyzing the characteristics of HDV patients from different regions worldwide, the authors stated that men are more frequently infected with HDV than women and

that regional differences concerning disease epidemiology and management exist (33).

The interaction between HBV and HDV is complex and multiple virological and host-related factors may be involved. HDV may be temporarily or permanently the dominant virus (34) and HBV DNA levels are often low or even undetectable in patients with chronic HDV infection (33, 35). Also, in the present study both anti-HDV positive cases were HBeAg negative and HBV DNA was undetectable, although the HBV genotype may play a role in the course of chronic hepatitis D. For instance, HBV genotype C has been associated with adverse outcomes (cirrhosis, hepatocellular carcinoma, or mortality) in patients with chronic hepatitis D (36). In Cuba, HBV genotypes A and D are prevalent (9). Since these two genotypes seem to have a very different influence on HDV infectivity (37), it would be interesting to know with which HBV genotype HDV positive patients are infected to anticipate disease outcome.

For the HDV-PCR positive patient, RNA was undetectable after 1 year of Interferon treatment and the liver enzymes were normal suggesting a virological and biochemical response. Although this is the first choice of HDV treatment and decreases the HDV viral load in most patients, only 25% of the patients have undetectable levels of RNA afterward (1, 6, 38, 39). A follow up is needed to verify whether the treatment has a sustained virological response over time, even beyond 24 weeks after treatment suspension (1). Most patients with HBV/HDV coinfection have high levels of ALT, AST and TSB and maintain a stable condition for a long time before decompensation or hepatic carcinoma occur (8, 33, 40). Disease progression may be influenced by the HDV genotype, with types 1 and 3 being linked to a more severe disease than genotypes 2 and 4 (6, 35, 40).

Conclusion

This is the first HDV study, including molecular detection and virus characterization, done after the introduction of the universal childhood anti-hepatitis B vaccination. The very low prevalence of HDV infection in HBsAg carriers combined with the high HBV vaccination coverage of all newborn children, of previously identified risk groups, and of the general population currently under 40 years of age, suggest that HDV elimination is feasible in Cuba if the success in HBV control is maintained.

Data availability statement

The data presented in this study are deposited in the Genbank repository (https://www.ncbi.nlm.nih.gov/genbank/), accession number: MW273290.

Ethics statement

The study was conducted in compliance with the Declaration of Helsinki and using Good Laboratory Practices. The specimens tested for this research were residual samples received for HBV serological and/or molecular analysis. The research was approved by the Ethics Committee of the Institute for Tropical Medicine in Havana, Cuba (CEI-IPK 05-16). In case of positive results, the doctor in charge was informed. Written informed consent was obtained from the patient with active HDV infection (HDV-RNA positive) for reviewing the clinical history and for taking serum samples for the follow-up of the HBV and HDV infection status.

Author contributions

LÁ and JH: conceptualization, resources, supervision, and project administration. LÁ, MV, ZT, MC, DH, BS, MS, LA, and AS: methodology. LÁ, AS, and JH: software. LÁ, MV, MS, and AS: validation. LÁ, MV, MS, ZT, MC, DH, BS, AS, and JH: analysis. LÁ, MS, and ZT: investigation. LÁ, MS, and LA: data curation. LÁ: writing—original draft preparation. LÁ, MS, and JH: writing—review and editing. LÁ, MS, AS, and JH: visualization. JH: funding acquisition. All authors read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Hepatitis B virus and hepatitis D virus infection in women with or at risk for HIV infection in the United States

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Hepatitis D virus (HDV) requires co-infection with hepatitis B virus (HBV). Human immunodeficiency virus (HIV) shares transmission routes with these viruses. Among 4,932 US women infected with or at-risk for HIV during 1994–2015, HBV surface antigen (HBsAg) positivity was more common in women with HIV (2.8% vs. 1.2%; p=0.001); HDV was more common among participants enrolled during 2013–2015 (p=0.0004) and those with resolved rather than active hepatitis C (1.9% vs. 0.5%; p=0.02). Among HBsAg-positive women (n=117), HDV antibody prevalence was 22% and did not vary by HIV status; HDV infection was associated with the presence of advanced fibrosis/cirrhosis at enrollment (adjusted odds ratio, 5.70; 95% confidence interval, 1.46–22.29). Our results demonstrate the importance of HDV testing in HBV-infected US women.

KEYWORDS

epidemiology, hepatitis B virus, hepatitis D virus, hepatitis C virus, anti-HDV, persons who injected drugs

Background

Hepatitis D virus (HDV) is a defective subviral pathogen that requires hepatitis B virus (HBV) for replication. An estimated 357,000 people in the United States are believed to have had past or ongoing HDV infection, although prevalence data are limited due to a paucity of previous studies (1).

HBV/HDV coinfected individuals experience more rapid progression to liver cirrhosis, hepatic decompensation, hepatocellular carcinoma, and death compared to those infected with HBV alone (2). HBV and HDV can be transmitted both sexually and through parenteral exposure. Given shared routes of transmission with human immunodeficiency virus (HIV) infection, people living with HIV (PLWH) or at risk for HIV infection are at higher risk for acquiring HDV. Furthermore, HDV-related liver disease may progress faster in PLWH, significantly impacting quality of life and survival (3). However, little is known about the prevalence of HDV infection in this population in the United States (4). With new, effective treatments for HDV potentially on the horizon (5, 6), it is important to determine HDV prevalence in high-risk populations, such as PLWH or at risk for acquiring HIV infection, to inform HDV screening and treatment.

The Women's Interagency HIV Study (WIHS) is a large prospective study designed to investigate the treatment and prevention of HIV infection among US women. Using the HDV quantitative microarray antibody capture (Q-MAC) assay, we examined the prevalence of HDV infection among the WIHS participants overall and in subgroups defined by demographic, behavioral and clinical characteristics, including HIV infection status.

Methods

Study population

WIHS is a multi-center cohort study that was established in 1994 to investigate the natural history and treatment of HIV infection and associated morbidities among women living in the United States. Details regarding study methods and follow-up protocols have been previously described (7). WIHS investigators recruited 3,677 HIV-seropositive women and 1,305 sociodemographically similar HIV-seronegative women at 10 U.S. study sites over 4 enrollment waves between 1994 and 2015. Data are collected prospectively through semiannual physical examinations, biological specimen collections and structured interviewer-administered questionnaires to obtain information regarding sociodemographic and risk behaviors including whether the participant had ever injected drugs or participated in transactional sex (defined as exchange of sex for drugs, money, or shelter). In 2019, the WIHS became part of the MACS/WIHS Combined Cohort Study (MWCCS) with most women continuing enrollment under a similar protocol (8).

Laboratory methods

Testing was performed on blood specimens collected at "baseline" (study entry for 99.8% of participants). As previously described (9), assays from Abbott Laboratories were used to detect hepatitis B surface antigen (HBsAg; Auszyme Microparticle enzyme immunoassay [EIA]). We considered women who tested positive for HBsAg at baseline to have "active HBV infection" at that time point. In these participants, we used HBsAg test results from subsequent visits, performed using the Seimens ADVIA Centaur Immunoassay System, and classified "chronically infected" participants as those with a second positive HBsAg result at least 180 days after the initial positive result.

Hepatitis C (HCV) antibody status was assessed in baseline samples using enzyme immunoassay (EIA) 2.0 [Abbott Laboratories] and 3.0 [Ortho-Clinical Diagnostics] with testing for HCV RNA using either the COBAS Amplicor HCV Detection Kit, COBAS Taqman Assay [Roche Diagnostics] or Quantiplex 2.0 branched chain DNA-enhanced label amplification assay [Bayer-Versant Diagnostics] in participants who were positive for HCV antibody. We defined HCV status as "never infected" for participants who tested negative for HCV antibodies, "resolved infection" for those who tested positive for anti-HCV but negative for HCV RNA and "actively infected" for those who tested positive for HCV RNA. HIV infection status was based on results of an enzyme-linked immunosorbent assay with western blot confirmation [NASBA/NuciSens HIV RNA assay, BioMerieux] (10).

Baseline measures of aspartate aminotransferase (AST) levels and platelet counts were determined using standard laboratory protocols. APRI was calculated as (100 x [AST/AST ULN]/platelet count [10^9 /l]) (11).

Because HDV replication requires HBsAg, we limited testing for HDV to reposited baseline samples from participants with active HBV infection. As previously described (12), HDV Q-MAC is a high-throughput assay with high sensitivity and specificity for detecting anti-HDV immunoglobulin G (12, 13). After excluding two participants without an available specimen, we tested 117 HBsAg-positive participants for anti-HDV.

Statistical analysis

Among women who were tested for HBsAg, we examined the prevalence of active HBV infection and the prevalence of HDV infection. We also determined anti-HDV seroprevalence among participants with active HBV infection. To assess whether infection prevalence differed in subgroups, we examined prevalence in strata defined by demographic, behavioral and clinical characteristics. Prevalence estimates were compared by χ^2 test or Fisher's exact test (when ≥25% of cells had a count <5).

Unconditional logistic regression was used the assess the association between HDV infection and selected variables, including year of enrollment, transactional sex, HCV status, injection drug use, and HIV infection. Multivariable analyses were not possible due to sparse data for some of those variables.

Polytomous logistic regression models were used to estimate the odds ratio (OR) and 95% confidence interval (CI) for associations between HDV infection and the aspartate aminotransferase to platelet ratio index (APRI). Measures of APRI were categorized in the following manner: no significant fibrosis (<0.5), significant fibrosis (0.5 to <1.0), advanced fibrosis/cirrhosis (≥1.0). Age at baseline, HCV status (dichotomized as actively infected vs. never infected/resolved infection) and HIV status were considered as potential confounders.

All analyses were performed with SAS software version 9.4 (SAS Institute, Inc.)

Results

Among the entire WIHS cohort of 4,982 women, 4,932 (99.0%) were tested for HBsAg at baseline. The median age at enrollment for the overall cohort was 36 years, 2,588 (52.5%) of participants were recruited between 1994 and 1995, and 3,033 (61.5%) of the women were non-Hispanic Black (Table 1). At baseline, 1295 (26.3%) reported ever having injected drugs, 1713 (34.7%) reported a history of transactional sex, 1,045 (21.2%) were actively infected with HCV, and 3,634 (73.7%) were HIV-positive. Women with active HBV infection (i.e., HBsAg-positive at baseline) represented 2.4% of the analytic cohort.

We examined the prevalence of active HBV infection, by demographic, behavioral and clinical characteristics (Supplemental Table 1). HBV infection was more common in women who enrolled in 1994–95 (66 [2.6%]) or 2013–15 (30 [3.6%]) than those who entered the study during 2001–02 (16 [1.4%]) or 2011–12 (5 [1.4%]). HBV infection was not significantly more common in women who reported injection drug use (36 [2.8%] vs. 81 [2.2%]; $p = 0.27$) but was found more often in women who reported engaging in transactional sex (56 [3.3%]) than those who did not (61 [1.9%]; $p = 0.004$). Active HBV infection was more common in women with resolved HCV infection (19 [5.2%]) than those with either active HCV infection (20 [1.9%]) or those who had never been infected (78 [2.2%]). Active HBV was present in 102 (2.8%) of women with HIV infection compared to 15 (1.2%) of uninfected women ($p = 0.001$). 85 (72.6%) participants had subsequent testing for HBsAg (median time to repeat testing, 6.9 years). The repeat test was positive for 47 (55.3%), who presumably had chronic hepatitis B, and negative for 38 (44.7%), who could have had acute HBV infection at study entry or chronic infection followed by loss of HBsAg either spontaneously or due to treatment.

Among WIHS participants overall, 26/4932 (0.5%) were positive for anti-HDV (Table 2). Anti-HDV prevalence did not differ by age, but participants who entered the cohort in 2013–2015 were more likely to be anti-HDV positive compared to those who enrolled in 1994–95 (13 [1.5%] vs. 10 [0.4%]; $p = 0.0004$). With the exception of one participant whose country of birth was missing, all anti-HDV seropositive participants were born in the United States. Women who reported injection drug use or transactional sex had a higher prevalence of anti-HDV seropositivity, although those differences were not statistically significant. Compared to the women who enrolled in 1994–1995, the participants who enrolled in 2013–2015 were less likely to report injection drug use (61 [7.2%] vs. 1,034 [40.0%]) and equally likely to report transactional sex (314 [37.1%] vs. 951 [36.9%]). Women with resolved HCV infection were approximately four times more likely to have anti-HDV seropositivity than those with active HCV infection (7 [1.9%] vs. 5 [0.5%], respectively; $p = 0.02$). There was no significant difference in HDV-seropositivity between those who were never infected with HCV and those with active HCV infection (14 [0.4%] vs. 5 [0.5%], respectively; $p = 0.78$). The prevalence of anti-HDV seropositivity was 0.6% (95% CI: 0.4–0.9) among women with HIV infection and 0.4% (95% CI: 0.2–0.9) in those who were HIV negative.

Among the 117 women with active HBV infection who were tested for anti-HDV, 26 (22.2%) were positive for HDV antibody (Table 2). HDV prevalence increased with age at enrollment. While a test for trend in age categories was not significant, median age was 39.0 years for HDV-positive women and 34.0 years for HDV-negative women ($p = 0.02$). As per the overall cohort, among women with active HBV infection, anti-HDV seropositivity was significantly

TABLE 1 Demographic, behavioral, and clinical characteristics of women screened for HBsAg at enrollment in the WIHS cohort.

Characteristic	Full Cohort[‡]	HBsAg+
Sample size, N (%)	4,932	117
Median Age (IQR), years	36 (30–43)	35 (30–40)
Year of Enrollment		
1994–95	2,588 (52.5)	66 (56.4)
2001–02	1,142 (23.2)	16 (13.7)
2011–12	356 (7.2)	5 (4.3)
2013–15	846 (17.2)	30 (25.6)
Race/Ethnicity, N (%)		
White (non-Hispanic)	658 (13.3)	12 (10.3)
White (Hispanic)	348 (7.1)	5 (4.3)
Black (non-Hispanic)	3,033 (61.5)	87 (74.4)
Black (Hispanic)	110 (2.2)	2 (1.7)
Other (Hispanic)	625 (12.7)	10 (8.5)
Asian/Pacific Islander	49 (1.0)	0 (0.0)
Native American/Alaskan Native	37 (0.8)	1 (0.9)
Other	72 (1.5)	0 (0.0)
Country of Birth		
United States	3,953 (80.2)	98 (83.8)
Other	947 (19.2)	14 (12.0)
Missing	32 (0.6)	5 (4.3)
Injection Drug Use, N (%)		
Yes	1,295 (26.3)	36 (30.8)
No	3,637 (73.7)	81 (69.2)
Transactional sex, N (%)		
Yes	1713 (34.7)	56 (47.9)
No	3,205 (65.0)	61 (52.1)
Missing	14 (0.28)	0 (0.0)
HBsAg, N (%)		
Positive	117 (2.4)	117 (100%)
Negative	4,813 (97.6)	0 (0%)
HCV Status, N (%)		
Active	1,045 (21.2)	20 (17.1)
Resolved	367 (7.4)	19 (16.2)
Never Infected	3,512 (71.2)	78 (66.7)
Missing	8 (0.2)	0 (0.0)
HIV, N (%)		
Positive	3,634 (73.7)	102 (87.2)
Negative	1,298 (26.3)	15 (12.8)

HCV, hepatitis C virus; HIV, human immunodeficiency virus; HBsAb, hepatitis B surface antibody; IQR, interquartile range. [‡]2 HBsAg + participants were excluded from analysis due to lack of serum for HDV testing.

TABLE 2 Prevalence of HDV antibody among WIHS participants, by selected demographic, behavioral and clinical characteristics.

Characteristics	Anti-HDV positive	All participants			Active HBV infection (HBsAg positive)		
		Total no.	Prevalence of anti-HDV, % (95%CI)[b]	*p*-Value[a]	Total no.	Prevalence of anti-HDV, % (95%CI)[b]	*p*-Value[a]
Overall	26	4,932	0.5 (0.4–0.8)	–	117	22.2 (15.6–30.6)	–
Age at enrollment, years[c]				0.52			0.16
16–29	4	1,123	0.4 (0.1–0.9)		27	14.8 (5.9–32.5)	
30–39	10	2026	0.5 (0.3–0.9)		57	17.5 (9.8–29.4)	
40–49	10	1,289	0.8 (0.4–1.4)		28	35.7 (20.7–54.2)	
≥50	2	494	0.4 (0.1–1.5)		5	40.0 (11.8–76.9)	
Race/Ethnicity, N (%)*							
White (non-Hispanic)	3	658	0.5 (0.2–1.3)	(ref)	12	25.0 (8.9–53.2)	(ref)
Black (non-Hispanic)	23	3,033	0.8 (0.5–1.1)	0.61	87	26.4 (18.3–36.6)	1.00
Country of Birth							
United States	25	947	2.6 (1.7–3.9)	–	98	25.5 (17.2–35.3)	–
Other	0	3,953	–	–	14	–	–
Year of Enrollment							
1994–95	10	2,588	0.4 (0.2–0.7)	(ref)	66	15.2 (8.4–25.7)	(ref)
2001–02	3	1,142	0.3 (0.1–0.8)	0.77	16	18.8 (6.6–43.0)	0.72
2011–12	0	356	–	–	5	–	–
2013–15	13	846	1.5 (0.9–2.6)	0.0004	30	43.3 (27.4–60.8)	0.02
Injection Drug Use							
Yes	10	1,295	0.8 (0.4–1.4)	0.16	36	27.8 (15.9–44.0)	0.45
No	16	3,637	0.4 (0.3–0.7)	(ref)	81	19.8 (12.5–29.7)	(ref)
Transactional Sex							
Yes	13	1713	0.8 (0.4–1.3)	0.15	56	23.2 (14.1–35.8)	0.84
No	13	3,205	0.4 (0.2–0.7)	(ref)	61	21.3 (12.9–33.1)	(ref)
HCV Status							
Active	5	1,045	0.5 (0.2–1.1)	(ref)	20	25.0 (8.7–49.1)	(ref)
Resolved	7	367	1.9 (0.8–3.9)	0.02	19	36.8 (16.3–61.6)	0.74
Never Infected	14	3,512	0.4 (0.2–0.7)	0.78	78	18.0 (10.2–28.3)	0.55
HIV							
Positive	21	3,634	0.6 (0.4–0.9)	0.41	102	20.6 (13.9–29.4)	0.37
Negative	5	1,298	0.4 (0.2–0.9)	(ref)	15	33.3 (15.2–58.3)	(ref)

*HDV infection was detected only in women of White (non-Hispanic) or Black (non-Hispanic) "Race/Ethnicity".

CI, confidence interval; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; HBsAg, hepatitis B surface antigen.

[a]*p*-value from chi-square test unless indicated otherwise.

[b]Exact binomial CIs.

[c]*p*-value for trend from univariate logistic regression for each category increase of age.

Prevalence estimates among subgroups were compared by Chi-squared test or Fisher's exact test, as appropriate. All tests were two-sided and *p*-values < 0.05 were considered statistically significant.

higher in those who enrolled in 2013–2015 (13 [43.3%]) than those who enrolled in 1994–95 (10 [15.2%]; p = 0.02; OR, 4.3; 95% CI, 1.6–11.5; Supplemental Table 2). Anti-HDV seropositivity prevalence was higher in women who reported injection drug use (10 [27.8%]) than those that did not (16 [19.8%]), but that difference was not statistically significant ($p = 0.45$). Among women with active HBV infection, antibody to HDV was present in 21 (20.6%) of the women with HIV infection, compared to 5 (33.3%) who were not infected with HIV ($p = 0.37$).

We examined the relationship between HDV infection, and the presence of fibrosis as defined by APRI-based categories among the participants with active HBV. Compared to women who had no significant fibrosis, those with advanced fibrosis/cirrhosis where much more likely to be positive for HDV, (OR = 6.00; 95%CI: 1.61–22.42) (Table 3). After adjusting for age at baseline, this association was slightly attenuated (adjusted OR = 5.70; 95%CI: 1.46–22.29). HCV status did not confound this relationship. We were unable to assess potential confounding by HIV status because all participants with

TABLE 3 Odds ratios (ORs) and 95% confidence intervals (CIs) for the association between HDV and fibrosis category (based on APRI values at enrollment) among WIHS participants with active HBV infection (n=117).

	APRI		
	OR (95%CI)		p-value*
	Significant fibrosis vs. no significant fibrosis	Advanced fibrosis/cirrhosis vs. no significant fibrosis	
Unadjusted	1.88 (0.62, 5.63)	6.00 (1.61, 22.42)	0.03
Adjusted for age at baseline	1.72 (0.55, 5.33)	5.70 (1.46, 22.29)	0.04

[d]APRI categories: "no significant fibrosis", <0.5 (N=84); "significant fibrosis", 0.5 to < 1.0 (N=22); "advanced fibrosis/cirrhosis", ≥1.0 (N=11).
*value of ps calculated as p-trend.

advanced fibrosis/cirrhosis were HIV-positive. No association was found between HDV status and significant fibrosis when compared to no significant fibrosis (OR = 1.88; 95%CI: 0.62–5.63).

Discussion

We evaluated the prevalence of anti-HDV seropositivity in a cohort of US women with or at risk of HIV infection. Using an assay with excellent performance characteristics for detecting anti-HDV, 22% of women with active HBV infection had evidence of HDV infection. While the women with HIV had a significantly higher prevalence of active HBV infection, anti-HDV prevalence did not differ by HIV status, although power was limited.

While anti-HDV seroprevalence was common among HBsAg-positive women enrolled in WIHS, the prevalence was lower than that reported in previous studies. Among people who inject drugs enrolled in the Urban Health Study (UHS), we found that 35.6% of those with active HBV infection were positive by the HDV QMAC assay (13). Other studies of HBV/HDV co-infection among individuals in the US who inject drugs reported prevalence estimates from 42 to 67% (14–16). In the 2011–2016 National Health and Nutrition Examination Survey, which is based on the general US population, investigators reported anti-HDV prevalence was 42% in those with active HBV. (1) Reported differences in anti-HDV prevalence may reflect differences in population, associated risk factors and test characteristics of anti-HDV assays. A limitation of this study is the lack of data on HDV RNA; nevertheless, in a previous study conducted in the United States by our group, all participants who tested positive by QMAC was also found to be positive for HDV RNA (13).

Anti-HDV seroprevalence was highest among the women who enrolled during 2013–2015, which is the latest period of enrollment. However, that finding should be interpreted with caution because WIHS enrollment in 2013–2015 was almost exclusively from southern U.S. states, whereas participants from other regions predominated in earlier enrollment waves. The higher prevalence of anti-HDV among the most recent WIHS enrollees thus could reflect either temporality or geography. To our knowledge, prevalence by geographical region has not been assessed in national surveys of HDV infection in the United States (1) and might be the subject of future studies.

Interactions between hepatitis viruses affect viral clearance (13). In the cohort overall, active HBV infection was more common in WIHS participants with resolved hepatitis C than those with active HCV infection, or those who had never been infected with HCV. These results correspond with our observations in UHS (13). In the cohort overall, anti-HDV prevalence was lower in those with active HCV infection than in those with resolved HCV infection. Among HBsAg+ positive participants, there were no meaningful difference in HDV prevalence by HCV status, whereas in UHS, we saw a slightly higher HDV prevalence in HBsAg+ participants with resolved compared to chronic HCV infection (13). Due to the cross-sectional design of these studies, temporality of viral acquisition could not be assessed, nevertheless, these results suggest that active HBV suppressed HCV infection in these populations. Longitudinal studies are needed to clarify interactions among hepatitis viruses.

In accordance with previous studies (2, 17), we found an association between HDV status and advanced fibrosis/cirrhosis at enrollment. Although we were limited by our lack of data on duration of infection, adjusting for age at baseline as a proxy measure still yielded a significant association between HDV status and advanced fibrosis/cirrhosis as defined by APRI.

Current guidelines for HDV testing recommend that persons belonging to certain 'high-risk' groups (including those with HIV or HCV infection, persons who have ever injected drugs, individuals with multiple sex partners) who test positive for HBsAg should also be tested for HDV infection (18). If we consider a history of transactional sex as an indicator of multiple sex partners, 89.7% of the women who were positive for HBsAg and 88.4% of the women who were positive for HDV had at least one risk factors that should prompt clinical testing for HDV. Data on previous HDV testing in clinical practice is not available for WIHS participants.

Our results provide additional evidence that HDV is common among individuals with active HBV infection in the US and support current recommendations that persons belonging to 'high-risk' groups who test positive for HBsAg should be tested for HDV infection (18). Little is known about the uptake of HDV screening recommendations in the United States. Future efforts should evaluate HDV testing rates in the US and seek to raise awareness regarding the importance of screening for this highly pathogenic virus.

Data availability statement

Access to individual-level data from the MACS/WIHS Combined Cohort Study Data (MWCCS) may be obtained upon review and approval of a MWCCS concept sheet. Links and instructions for online concept sheet submission are on the study website (https://statepi.jhsph.edu/mwccs/work-with-us/).

Author contributions

IA, PM, RP, and TO'B contributed to the conception and design of the study. PL and JG conducted laboratory analyses. AA, MA, HB, AF, MP, JP, AR, AS, ES, and MK manage the cohort and corresponding data. IA conducted the statistical analyses and took the lead in writing the manuscript. All authors provided critical feedback and review of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sequence diversity of hepatitis D virus in Mongolia

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Introduction: The Hepatitis Delta Virus (HDV) is a defective, single-stranded RNA virusoid encoding for a single protein, the Hepatitis Delta Antigen (HDAg), which requires the hepatitis B virus (HBV) envelope protein (HBsAg) for its transmission. Currently, hepatitis D is the most aggressive form of viral hepatitis and treatment options are limited. Worldwide 12 million people are chronically infected with HDV being at high risk for progression to cirrhosis and development of liver cancer.

Objectives: Although it is well established that Mongolia is the country with the highest prevalence of HDV infections, the information on the molecular epidemiology and factors contributing to HDV sequence diversity are largely unclear. The aim of the study was to characterize the sequence diversity of HDV in rural areas from Mongolia and to determine the extent of HLA class I-associated selection pressure.

Patients and methods: From the HepMongolia cohort from rural areas in Mongolia, 451 HBsAg-positive individuals were selected and anti-HDV, HDV-RNA and the sequence of the large HDAg was determined. For all individuals the HLA class I locus was genotyped. Residues under selection pressure in the presence of individual HLA class I types were identified with the recently published analysis tool HAMdetector.

Results: Of 431 HBsAg positive patients, 281 were anti-HDV positive (65%), and HDV-RNA could be detected in 207 of 281 (74%) of patients. The complete large HDAg was successfully sequenced from 131 samples. Phylogenetic analysis revealed that all Mongolian HDV isolates belong to genotype 1, however, they separate into several different clusters without clear regional association. In turn, from phylogeny there is strong evidence for recent local transmission events. Importantly, we found multiple residues with strong support for HLA class I-associated selection pressure consistent with a functional $CD8^+$ T cell response directed against HDV.

Conclusion: HDV isolates from Mongolia are highly diverse. The molecular epidemiology suggests circulation of multiple subtypes and provides evidence for ongoing recent transmissions.

KEYWORDS

sequence diversity, hepatitis D virus, Mongolia, HLA class I-associated selection pressure, molecular epidemiology, HDV subtypes

Introduction

Hepatitis delta virus (HDV) infection causes the most severe form of viral hepatitis including faster progression toward liver-related death and hepatocellular carcinoma (1). HDV is globally prevalent, although prevalence varies greatly between countries. According to current estimates from a meta-analysis by Stockdale et al. (2020), approximately 12 million people worldwide are anti-HDV positive (2). Mongolia has the highest national anti-HDV prevalence among those testing positive for HBsAg [ranging from 35% in the global population up to 83% in high risk groups in Ulaanbaatar City (1–3)], followed by Moldova and countries in West and Central Africa (>10%) (2). Currently, the only treatment options are peginterferon alfa-2a and the entry inhibitor bulevirtide (4, 5). Unfortunately, long-term sustained HDV RNA negativity is only rarely achieved in patients treated with peginterferon alfa-2a (6, 7). Bulevirtide induces a decline in HDV RNA during treatment, however, after cessation of bulevirtide HDV RNA rapidly rebounds (6). Studies with longer treatment durations and combination of both therapies are ongoing and might result in higher cure rates (4).

HDV is a satellite virus that requires hepatitis B virus (HBV) for its virus assembly and propagation. HDV has a single-stranded, circular RNA genome approximately 1,700 nucleotides in length, that is replicated by rolling circle amplification (8). HDV virions consist of a nucleocapsid-like ribonucleoprotein (RNP), in which the HDV RNA is associated with the hepatitis D antigen (HDAg), that is enveloped by HBV surface antigen (HBsAg) (8). RNA genome editing of an amber stop codon at position 196 by a cellular adenosine deaminase (ADAR) leads to production of the large isoform with 215 aa, whereas the unedited genome produces the 196 aa isoform (9). Both isoforms undergo extensive post-translational modifications to fulfill diverse functions in genome replication, HDV RNP assembly and HDV RNP packaging by the HBsAG and inhibitors of post-translational modifications, such as lonafarnib, are currently evaluated in clinical trials (10).

HDV can be grouped into eight distinct genotypes and possibly multiple subtypes (11, 12). In a recent study, criteria for the definition of subtypes have been suggested along with a set of reference sequences for subtyping of HDV genotype 1 isolates (11). These genotypes and subtypes associate with specific global and regional distribution (3, 12, 13). The overall extent of HDV sequence diversity especially at the subtype level is not well defined. This is important information for studies of the molecular epidemiology of HDV both globally, but also locally for detection of possible transmission chains by sequence analysis. A database of HDV sequences was recently established with so far about 1,000 complete sequences of the large HDAg (14). Notably, the mechanisms driving the sequence variation of HDV have so far not been studied in detail. In recent studies, it has been shown also for HDV that $CD8^+$ T cells contribute to sequence variation by selecting mutations in targeted epitopes (15, 16). HLA class I-associated selection pressure on HDV was also documented at the population level (17). Accordingly, viral sequence analysis in concert with HLA class I genotyping opens up the opportunity to detect novel epitopes under $CD8^+$ T cell selection pressure. Given the sparsity of our knowledge about the targeted epitopes in HLA-diverse populations, this is highly relevant for studies in HDV immunology (18, 19).

In Mongolia, viral hepatitis is an enormous public health problem. Chronic infections with HBV or HCV are highly prevalent, each affecting between 10% and 20% of the population (3, 20, 21). Importantly, co-infections with HDV are also highly prevalent in Mongolia (3, 21, 22). This high rate of chronic hepatitis creates an immense health burden of advanced liver disease associated with liver failure and is the cause for the highest incidence rate of hepatocellular carcinoma worldwide (23–26). Despite the high number of HDV infected patients only few studies performed HDV sequence analysis (12, 27) and information on the molecular epidemiology and the extent of HDV sequence diversity of Mongolian virus isolates is not well defined (3). Furthermore, to our knowledge, there are no studies analyzing the effect of host-genetics and selection pressure on virus variability in Mongolia. To analyze the phylogenetic relationship of Mongolian HDV sequences and the influence of host-genetics on HDV sequence diversity of HDV we set up the HepMongolia cohort. For this convenience sampling cohort, people with self-reported liver disease were recruited in different areas from Mongolia. People were offered an HBsAg rapid test and individuals tested positive were included into this study.

Materials and methods

Patients

Samples of patients with HBV- or HBV/HDV-infection were obtained within the HepMongolia study for the surveillance of hepatitis in rural areas in Mongolia (Supplementary Table 1). Blood samples were collected from patients with self-reported liver disease from soums (small villages or small administrative regions within Mongolian provinces) in Western and Central Mongolia. A highly sensitive point-of-care-test for HBsAg (Onsite HBsAg Combo Rapid Test, #R0042C, CTK Biotech Inc., Poway, CA, United States; sensitivity 100, 95% CI [95.9%–100%]; specificity 98.3, 95% CI [95.2%–99.4%] (28)) was performed on site and HBsAg-positive patients were further studied. Written informed consent was obtained from all study participants and the study was approved by the Ethics Committee of the Mongolian Ministry of Health (study #2018-79-MEIC) and Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf, Germany (#2019-404-KFogU).

Serology

Serology was done in the routine diagnostic of the Institute of Virology Düsseldorf. HBsAg was detected and quantified with the HBsAg qualitative II Kit (#2G22-30) and the HBsAg quantitative (#6C36-43) on an Abbot ARCHITECT i2000SR (all Abbot). HDV serology was done on a Liaison-XL, (DiaSorin) using the XL Murex Anti-HDV Kit (#311260).

Extraction of viral RNA and RT-PCR

Viral RNA from 400 µL plasma was extracted automatically using the EZ1 Virus Mini Kit v2.0 on an EZ1 Advanced XL robot or manually with the QIAamp Viral RNA Mini Kit (both Qiagen)

according to the manufacturer's protocol. RNA was eluted in a volume of 60 µL and stored at −80°C. RT-qPCR was performed with primer and probes from Mederacke et al. (29), however using the AgPath-ID One Step RT-PCR-Kit (Applied Biosystems, #4387424). To reduce hands on time, PCR mixes were prepared in large batches and frozen ("frozen-PCR mixes"). Per reaction, 25 µL RT-qPCR mixture containing 12.5 µL 2x RT-PCR-Buffer, 2.5 µL water, 1 µL of each primer (10 µM), HDV-Fwd-1 (TGGACGTKCGTCCTCCT; [positions 837 to 853]), HDV-Fwd-2 (TGGACGTCTGTCCTCCTT;[positions 837 to 854]), HDV-Rev (TCTTCGGGTCGGCATGG; [positions 891 to 907]) and 1 µL probe (10 µM) Delta-P (ATGCCCAGGTCGGAC; [positions 858 to 872]) were aliquoted in 8-strips and stored at −20°C until usage. For amplification, tubes were thawed and 5 µL of eluted viral RNA was used for RT-qPCR. Reverse transcription condition was 10 min at 45°C reverse transcription, 10 min at 95°C denaturation followed by 45 qPCR cycles each 15 s 95°C and 45 s annealing/extension at 60°C. The samples were quantified using a plasmid standard curve. The lower limit of detection was 75 copies per ml plasma and linearity was observed over the range of 1.5×10^3 to 3×10^8 copies/mL.

Amplification and sequence analysis of HDV

For amplification and sequencing, a modified protocol of Karimzadeh et al. (30) was used. For reverse transcription, a "primer-mix" containing 1 µL reverse primer HDV-771R (10 µM), 1 µL dNTPs (10 mM each) and 1 µL water was aliquoted in 8-strips with hinged-caps (Eppendorf, #951010022) and stored at −20°C until usage. For reverse transcription, an appropriate number of tubes was thawed and 10 µL HDV-RNA was added to the "primer-mix." Secondary RNA structures were melted for 5 min at 65°C and then samples were cooled down quickly to 25°C. RNA was reverse transcribed *in vitro* with Superscript III (SSIII, Invitrogen, #18080085) as previously described (31) by addition of 7 µL/well reverse transcription mix (4 µL SSIII-Buffer, 1 µL DTT, 1 µL RNase Inhibitor (NEB, #M0314L) and 1 µL SSIII) with the previously described conditions: 10 min at 25°C, 60 min at 42°C, 30 min at 50°C, 30 min 55°C, 15 min at 75°C and 4°C (31, 32). A two-step semi-nested PCR was performed with GoTaq HotStart-Polymerase (Promega, #M7401) according to the manufacturer's protocol and the following primer combinations for PCRI: HDV-891F (AGGTCGGACCGCGAGGAGGT); HDV-339R (GCTGAAGGGGTCCTCTGGAGGTG) and PCRII: HDV-912F (GAGATGCCATGCCGACCCGAAGAG); HDV-339R (GCTGAAGGGGTCCTCTGGAGGTG). Per reaction, 95 µL PCRI mixture containing 1x GoTaq polymerase buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs (Bio-Budget, #80–80,015,000), 0.5 µM of each primer and 1.25 units polymerase were mixed with 5 µL HDV-cDNA. PCR condition were 120 s at 94°C, followed by 45 cycles each 30 s 95°C, 30 s 64°C and 90 s 72°C followed by 10 min at 72°C and hold at 10°C. PCRII mixes were identical to PCRI except the final volume of 97 µL. Subsequently, 3 µL of the first round PCR-product was used for the second round of nested-PCR and the annealing temperature was set to 66°C with otherwise identical PCR conditions. PCR products were purified with the QIAquick PCR-Purification Kit (Qiagen, #28106) and Sanger sequenced with sequencing primer HDV-917F, HDV-339R and HDV-1419-Seq-F (TTCTTTCCGAGAATTCCTTTGA). Sequences were submitted to Genbank and are available under accession numbers (accession numbers: OQ024240–OQ024371).

Phylogenetic analysis of viral sequences and HAMdetector analysis

To analyze the genetic relationship and to provide the input files for the HAMdetector tool, all obtained sequences were aligned with the software Geneious 10.2.6 (RRID:SCR_010519) using MAFFT (33). For phylogenetic analysis a tree based on the large HDAg sequence, with references from Karimzadeh et al. (11), was calculated with the Mr. Bayes Plugin (34) in Geneious 10.2.8 using the GTR genetic distance model and GT3 as outgroup. For visualization the Posterior output was exported as Newick file with support values and visualized with itol (35).

HAMdetector is implemented as a julia package for identifying HLA associated substitutions based on aligned viral sequences paired to host HLA class I data. It integrates information from epitope prediction *via* MHCflurry 2.0 and phylogeny (based on RAxML-NG). The model is fit using Stan and the complete source code and documentation is available at https://github.com/HAMdetector/Escape.jl. For prediction, the large HDAg alignment used above was translated into an amino acid sequence. No adjustments were made to sequences where the amber-stop codon at position 196 was the majority. For phylogeny, the same nucleotide alignment was used as input.

HLA class I genotyping

For HLA genotyping, an amplicon-based approach using the Illumina next generation sequencing technology (Illumina Inc.) was used. Primers were designed to target exons 2, 3, and 4 for HLA class I genes HLA-A, -B and -C, as well as class I gene HLA-DPB1, and exons 2 and 3 for HLA-DRB1 and -DQB1. Amplicons comprise the entire exon and additional intronic sequences. All primers were screened for additional SNPs using the SNPCheck software[1] to avoid allele dropouts. Primers were purchased from Biolegio. Fragments were amplified in three multiplex PCR reactions. After clean-up using paramagnetic beads, sample-specific barcodes and Illumina compatible adapter sequences were added by PCR. Samples were pooled, underwent an additional purification step and were quantified using the QuantiFluor dsDNA system (Promega, # E2670). Seven pM of the NGS library were applied to the MiSeq instrument (Illumina Inc.) for a paired-end 2×280 cycles run using a standard v3 cartridge according to the manufacturer's instructions. As an internal quality run control, we used a spike-in of 15% PhiX. After de-multiplexing of the samples by the MiSeq Reporter software (Illumina Inc.), the analysis of the read sequences was performed by a self-developed software (NGSAnalyser) taking into account quality control values and high coverage to automate data analysis. Algorithms were developed to distinguish between sequencing artifacts such as cross-over products and closely related alleles. The American Society for

1 https://genetools.org/SNPCheck/snpcheck.htm

Histocompatibilty and Immunogenetics (ASHI) approved the entire NGS workflow including the self-developed software NGSAnalyser. The Institute of Transplantation Diagnostics and Cell Therapeutics (ITZ), University Hospital of Düsseldorf, Düsseldorf, is accredited to perform HLA typing for routine diagnostic purposes.

Results

Phylogenetic analysis of HDV isolates from Mongolia shows high sequence diversity and evidence for local transmission

In an approach to identify patients with HBV-infection in rural areas from Mongolia, individuals with self-reported liver disease were screened for HBsAg with a lateral-flow point-of-care test in 11 soums in 11 different provinces between 400 and 1,400 km West of Ulaanbaatar and one soum in the South East (Figure 1A). A total of 431 individuals with detectable HBsAg were identified (Table 1) of which 281 (65%) tested positive for anti-HDV (range 46%–73%). Of the 281 anti-HDV positive individuals, 207 (74%) had detectable HDV RNA in serum. The HDV RNA concentrations are shown in Figure 1B. The complete large HDAg (L-HDAg) was successfully sequenced from 131 individuals. In line with previous publications (27), phylogenetic analysis confirmed that all isolates were HDV genotype 1 (Figure 2A). Importantly, within the genotype 1 clade, sequences from Mongolia formed distinct phylogenetic clusters consistent with multiple subtypes. In a recent study, criteria for HDV subtyping were proposed and the authors suggested a reference set of sequences with assigned subtyping (11). These reference sequences were included in the phylogenetic analysis with the Mongolian sequences with the reference sequences color-coded for the five different subtypes 1a to 1e (Figure 2A). Notably, sequences from Mongolia clustered with subtypes 1c and 1d, however, the tree suggests further differentiation of isolates from Mongolia into so far unassigned subtypes. There are at least three additional highly reproducible clusters supported by bootstrap values >75%. As the samples were collected from different regions of Mongolia, we tested if the clusters associated with specific regions where samples were obtained (Figure 2B). However, samples collected from the same district were distributed across the whole tree and did not associate with specific clusters, strongly suggesting that regional transmission is not a major driver for these clusters. Interestingly, despite this overall high sequence diversity, there were also examples where two or three isolates have a particularly small genetic distance, indicating a common transmission event. Of note, in all these putative individual transmission events, samples were collected from the same soum consistent with local transmission. Taken together, there is overall high sequence diversity between HDV isolates collected in rural areas from Mongolia. The molecular epidemiology overall suggests regionally independent transmission of HDV, although on a local level there are individual examples strongly supporting a direct transmission event.

HLA-class I-associated selection pressure on the large HDAg contributes to the sequence diversity of HDV in Mongolia

Next, we analyzed if amino acid substitutions in the large HDAg are under positive selection in the presence of distinct HLA-molecules. It was previously described that HDV evades from the $CD8^+$ T cell response by selecting mutations in targeted epitopes (15, 17, 36, 37). These studies utilized cohorts from Europe or North America for analyses of $CD8^+$ T cell responses and the possible consequences of immune escape of HDV. We therefore addressed if similar mechanisms contribute to the HDV sequence diversity in Mongolia and whether the epitopes under selection pressure are shared across populations. We used the recently published tool HAMdetector (38) to probabilistically quantify the strengths and uncertainties of associations between HLA class I alleles and amino acid substitutions. The tool integrates several pieces of information that could contribute to an explanation of apparent HLA-substitution associations in a single coherent Bayesian regression model, with strong associations probably being the consequence of HLA class I selection pressure. The integrated pieces of information include: alignment of viral sequences, HLA alleles of patients, sparsity of HLA substitution associations, phylogeny of viral sequences, and possible HLA class I-binding motifs.

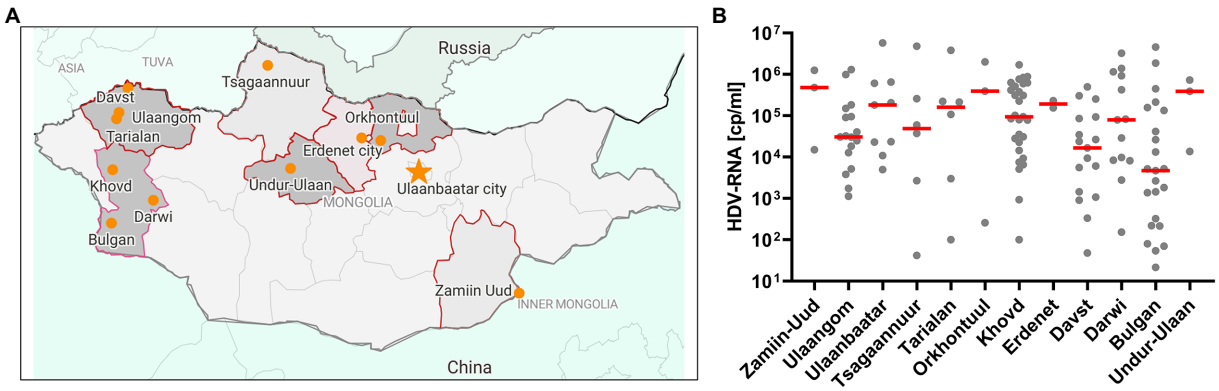

FIGURE 1
Recruitment of the HepMongolia cohort. **(A)** Sampling regions for the HepMongolia cohort. The map was created with data from open street map and visualized with datawrapper. **(B)** HDV viral load in the different sampling regions.

TABLE 1 HDV prevalence among the adult population of rural areas of Mongolia.

Province	Region	Size of settlements	HBsAg positive (*n*)	Female sex HBsAg positive *n* (%)	Median age HBsAg positive *n* (%)	Anti-HDV positive *n* (% of HBsAg)	HDV-RNA positive *n* (% of anti-HDV positive)	Female sex HDV-RNA positive *n* (%)	Median age HDV-positive
Arkhangai province	Undur-Ulaan	Soum (village)	13	6 (46)	49 (26–64)	6 (46)	4 (66)	2 (50)	53 (52–64)
Khovd province	Bulgan	Soum (village)	70	35 (49)	48 (15–86)	51 (73)	36 (71)	17 (47)	45 (21–64)
Khovd province	Darwi	Soum (village)	48	32 (64)	44,5 (16–61)	28 (58)	23 (82)	14 (60)	51 (37–61)
Uvs province	Davst	Soum (village)	51	21 (41)	46 (21–75)	37 (73)	24 (65)	10 (41)	49 (28–64)
Erdenet	Erdenet	City	15	5 (31)	32,5 (27–59)	10 (67)	7 (70)	2 (28)	33,5 (28–39)
Khovd province	Khovd	Provincial center	70	45 (64)	45 (23–70)	48 (69)	40 (83)	28 (70)	46 (25–62)
Selenge province	Orkhontuul	Soum (village)	18	12 (66)	42 (28–58)	11 (61)	7 (64)	3 (42)	52 (37–56)
Uvs province	Tarialan	Soum (village)	13	10 (76)	45 (28–69)	9 (69)	8 (89)	6 (75)	37 (32–60)
Khuvsgul province	Tsagaannuur	Soum (village)	18	8 (44)	43,5 (27–62)	12 (67)	9 (75)	5 (55)	40,5 (31–55)
Ulaanbaatar	Ulaanbaatar	Capital city	46	22 (47)	40,5 (32–58)	25 (54)	14 (56)	8 (57)	41 (33–58)
Uvs province	Ulaangom	Provincial center	62	38 (61)	46,5 (24–76)	40 (65)	31 (78)	20 (64)	50 (32–71)
Dornogovi province	Zamiin-Uud	Soum (village)	7	4 (57)	34 (26–48)	4 (57)	4 (100)	3 (75)	34 (32–44)
	Total		431	238 (55)	44 (18–86)	281 (65)	207 (74)	118 (56)	47 (21–71)

The resulting posterior probabilities of HLA substitution associations lie between 0 and 1 and can be interpreted easily. For instance, a posterior probability of 0 speaks strongly against an association, a value of 0.5 neither favors nor disfavors an association, and a value of 1 strongly supports an association and, hence, an HLA-selected mutation.

Viral sequence data and HLA class I genotypes were available for 131 individuals from the HepMongolia cohort. The HLA distribution of the HepMongolia cohort (Table 2) was comparable to previous studies from Mongolia (39–41). Notably, the extent of sequence variation at the amino acid level was not evenly distributed across the large HDAg. The Shannon entropy as a measure for the amino acid diversity at individual positions strongly varied including highly diverse as well as conserved residues (Figure 3A). This suggests that that different areas of the HDAg are subject to varying degrees of positive selection (e.g., by immune pressure) or negative selection (e.g., due to functional constrains).

Figure 3C shows the results of the analysis when HLA class I genotypes were randomly assigned to the viral sequences and serves as a control with no associations being expected. Only posterior probabilities > 0.5 are illustrated to focus on possible evidence for HLA class I-associated mutations. The majority of the posterior probabilities are below 0.7 with only few exceptions (Figure 3C; Supplementary Table 2). In contrast, when the true HLA class I genotypes were assigned to the viral sequences (Figure 3B), multiple substitutions with high posterior probabilities were detected. These substitutions are indicative for highly reproducible HLA class I-associated selection pressure on the L-HDAg in this cohort (Supplementary Table 3). In a previous study, there was experimental support for targeted $CD8^+$ T cell epitopes in HDV when posterior probabilities were above 0.8 (38). We therefore also used this cut-off to compare the results from the HepMongolia cohort to HDV isolates from Europe (38). According to these criteria, a total of 45 HLA class I-associated mutations were detected in 31 different positions of the large HDAg from Mongolia (Table 3). Interestingly, in only four positions the same or similar HLA-associated mutations were previously detected in a European cohort of patients with HDV infection suggesting that only some HAMs are shared across HLA-diverse populations. Two of these four shared HAMs were located inside previously described $CD8^+$ T cell epitopes restricted by the relevant HLA class I type. Of note, two additional HAMs were also located inside previously described $CD8^+$ T cell epitopes, however, here, no evidence for selection was detected in the European cohort.

The shared HAMs between both cohorts included the two substitutions with the highest posterior probability indicating selection pressure in the presence of HLA-B*37 and HLA-A*68 (Table 3). The exact frequencies of viral sequence polymorphisms in the HAM-containing region in the presence and absence of the relevant HLA class molecule are shown in Figure 4. In the previously described HLA-B*37-restricted epitope (QDHRRRKAL), substitutions are highly enriched at position 1 and 2 of the epitope (positions 100 and 101 of the L-HDAg) in the presence of HLA-B*37 but not in the absence, consistent with reproducible selection pressure in HLA-B*37-positive individuals (Figure 4A). In the context of

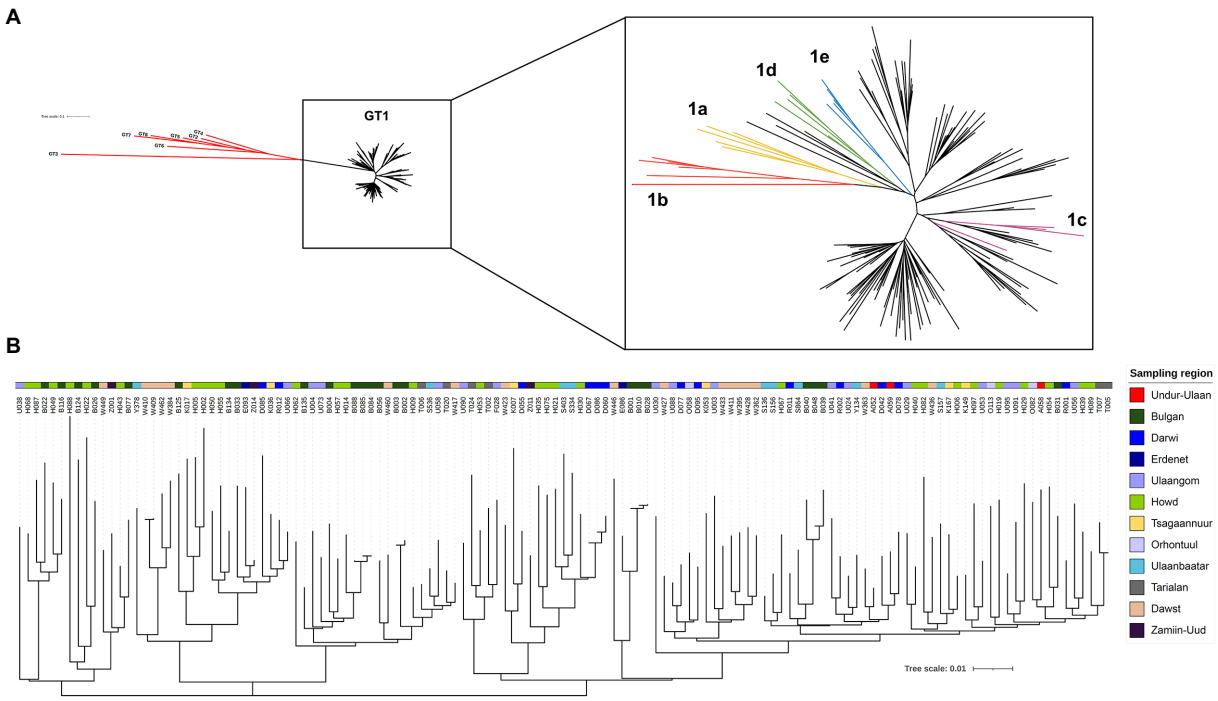

FIGURE 2
Phylogenetic analysis of viral sequences covering the large HDAg. A total of 131 HDV sequences covering the large HDAg were aligned with reference sequences [from (11)] using MAFFT. A phylogenetic tree was calculated with the Mr. Bayes Plugin (34) in Geneious 10.2.8 using the GTR genetic distance model and GT3 as outgroup. **(A)** *Left:* unrooted tree all sequences from the HepMongolia cohort together with reference sequences with assigned genotypes. Genotypes 2–8 are colored red, GT1 samples in black. *Right:* Inset showing only GT1 samples. Sequences from the HepMongolia cohort are colored black, GT1 subtypes described by [(11)] are color coded. **(B)** Disperse geographical distribution of HDV GT1 subtypes in Mongolia. The color code is according to the sampling region.

HLA-A*68, substitutions were highly enriched at position 81 of the large HDAg (Figure 4B). Although in the same position substitutions were also enriched in a European cohort, no $CD8^+$ T cell epitope has yet been confirmed. Notably, the HAM is located at position 2 of a peptide sequence that is predicted to bind to HLA-A*68 (Figure 4B). Importantly, the majority of HAMs in the Mongolian cohort are novel and may hint at novel, previously undetected epitopes. Figure 4C shows the region 13–25 of the large HDAg where the substitutions E15D and V16I are highly enriched in HLA-A*33 positive individuals. This region also encodes for a peptide sequence predicted to bind with high affinity to HLA-A*33 consistent with a novel epitope under selection pressure in Mongolia. Taken together, there is strong evidence that HLA-class I-associated selection pressure contributes to the sequence diversity of HDV in Mongolia.

Discussion

Mongolia is the country with the highest prevalence of viral hepatitis. The prevalence of HBsAg in the population is about 10%–12% with a decreasing trend in younger cohorts since the introduction of hepatitis B vaccination programs in 1991 (3). The reported percentage of anti-HDV positive individuals in Mongolian HBsAg carriers varies greatly with reported frequencies of 35% in the general population up to 83% in specific risk groups (2, 3, 21, 27). In our study, the proportion of anti-HDV positive individuals in HBsAg carriers was 65%. When recruiting patients for the study, persons with self-reported liver disease were targeted, which may explain an increased prevalence of HBV/HDV co-infection compare to the general population. HDV RNA was detected in 74% of the anti-HDV positive patients, which is in line with previous studies in populations with high anti-HDV prevalence (1, 42). Notably, the median age of people with HDV infection in this cohort was 47 years, with 30% of them being younger than 40 years suggesting still a high disease burden in younger cohorts. The main objective of the present study was to better characterize the sequence variability of HDV in Mongolia. For this purpose, the complete genomic region of large HDAg was successfully sequenced from 131 individuals. In the remaining HDV RNA-positive samples, sequencing was incomplete or unsuccessful, in most cases due to a relatively low viral load in the sample.

In the phylogenetic analysis, all isolates were assigned to genotype 1. In previous studies of HDV genotypes in Mongolia, genotype 1 was also the most common (2, 21, 27). Criteria for exact definitions of subtypes have not been established for HDV to date. Recently, criteria for subtyping HDV genotype 1 samples have been proposed and a dataset of reference sequences for the subtypes was published (11). Although some of the isolates from Mongolia clustered with reference sequences, no clear assignment to a subtype was possible for most isolates. With regard to the described subtypes, the isolates from Mongolia were most likely to be assigned to subtypes 1c and 1d, with subtype 1c dominating. Thus far, subtype 1c was mainly found in isolates from East Asia such as China, Vietnam or Japan, but also seems to occur frequently in Mongolia. Some isolates were most likely

TABLE 2 Comparison of the HLA polymorphism of the Mongolian population.

	HepMongolia (*n* =131;2022)	Mongolia Buryat (*n* =141; 2002)	Mongolia Khalkha (*n* =200;2002)	Mongolia Khalkha pop 2 (*n* =202;1995)	Mongolia Oold (*n* =104; 2002)	Mongolia Tarialan Khoton (*n* =85;1996)	Mongolia Tsaatan (*n* =144;2002)	Mongolia Ulaanbaatar Khalkha (*n* =41;1996)
HLA-A*01	0.084	0.114	0.080	0.075	0.144	0.066	0.125	0.085
HLA-A*02	0.302	0.501	0.265	0.279	0.327	0.094	0.243	0.192
HLA-A*03	0.046	0.036	0.060	0.030	0.058	0.079	0.069	0.038
HLA-A*11	0.122	0.075	0.100	0.106	0.077	0.107	0.076	0.069
HLA-A*23	0.015	0.015	0.015	0.005	0.039	0.000	0.021	0.012
HLA-A*24	0.183	0.246	0.195	0.208	0.183	0.214	0.222	0.231
HLA-A*26	0.046	0.014	0.060	0.067	0.058	0.094	0.028	0.049
HLA-A*29	0.023	0.008	0.010	0.010	0.010	0.048	0.000	0.000
HLA-A*30	0.015	0.011	0.020	0.021	0.000	0.082	0.021	0.037
HLA-A*31	0.057	0.083	0.085	0.070	0.058	0.065	0.049	0.049
HLA-A*32	0.008	0.046	0.020	0.010	0.000	nd	0.021	nd
HLA-A*33	0.065	0.004	0.060	0.050	0.039	0.000	0.125	0.089
HLA-A*68	0.034	0.029	0.015	0.015	0.000	0.000	0.000	0.026
HLA-B*07	0.042	nd	0.055	0.025	0.067	nd	0.034	nd
HLA-B*08	0.031	nd	0.035	0.052	0.010	nd	0.014	nd
HLA-B*13	0.069	nd	0.040	0.042	0.039	0.041	0.056	0.049
HLA-B*15	0.092	nd	0.115	0.057	0.067	0.036	0.111	0.077
HLA-B*18	0.008	nd	0.005	0.011	0.010	0.041	0.000	0.000
HLA-B*27	0.015	nd	0.025	0.010	0.058	0.006	0.021	0.000
HLA-B*35	0.069	nd	0.075	0.065	0.096	0.079	0.014	0.024
HLA-B*37	0.073	nd	0.035	0.042	0.029	0.018	0.069	0.066
HLA-B*38	0.015	nd	0.020	0.005	0.010	0.128	0.014	0.024
HLA-B*39	0.008	nd	0.010	0.010	0.000	0.006	0.000	0.000
HLA-B*40	0.103	nd	0.145	0.206	0.183	nd	0.174	nd
HLA-B*41	0.008	nd	0.000	nd	0.010	nd	0.014	nd
HLA-B*44	0.050	nd	0.040	0.030	0.048	0.094	0.028	0.049
HLA-B*46	0.019	nd	0.005	0.005	0.019	0.000	0.000	0.012
HLA-B*48	0.076	nd	0.035	0.057	0.039	0.035	0.021	0.073

(Continued)

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TABLE 2 (Continued)

	HepMongolia (*n* =131;2022)	Mongolia Buryat (*n* =141; 2002)	Mongolia Khalkha (*n* =200;2002)	Mongolia Khalkha pop 2 (*n* =202;1995)	Mongolia Oold (*n* =104; 2002)	Mongolia Tarialan Khoton (*n* =85;1996)	Mongolia Tsaatan (*n* =144;2002)	Mongolia Ulaanbaatar Khalkha (*n* =41;1996)
HLA-B*50	0.034	nd	0.020	0.035	0.010	nd	0.035	nd
HLA-B*51	0.084	nd	0.125	0.080	0.125	0.067	0.083	0.148
HLA-B*52	0.034	nd	0.025	0.020	0.039	0.071	0.049	0.037
HLA-B*54	0.038	nd	0.050	0.055	0.039	0.024	0.021	0.012
HLA-B*55	0.038	nd	0.025	0.015	0.019	nd	0.042	nd
HLA-B*56	0.015	nd	nd	0.020	nd	nd	nd	nd
HLA-B*57	0.015	nd	0.020	0.098	0.000	0.029	0.007	0.024
HLA-B*58	0.050	nd	0.070	nd	0.077	0.055	0.194	0.110
HLA-B*73	0.007633588	nd	0.005	nd	0	nd	0	nd

HLA-B*49 and HLA-B*67 had each only 1 participant and were therefore excluded.

to be assigned to subtype 1d, which was so far described predominantly in Turkey and Iran. Interestingly, in the phylogenetic analysis, further clusters were formed that cannot be clearly assigned to any of the described subtypes.

Analysis of the phylogeny of the viral sequences opened up the opportunity to study the molecular epidemiology of HDV in Mongolia. Importantly, there was no clear evidence for a regional clustering of the viral sequences. The sequences from samples collected in the same regions from Mongolia distributed across the whole tree. This is in line with the traditionally high mobility of people living in Mongolia. Especially in rural areas people are typically sedentary only for short periods of time. It is not well established when HDV was introduced and started to spread through the Mongolian population. The differentiation of HDV sequences from Mongolia in clusters may suggest that founder effects by introduction and spreading of different isolates contributed to the molecular epidemiology. However, additional studies in larger datasets will be required to address this in more detail.

Despite the lack of evidence for regional clustering of HDV isolates, there was strong evidence for local transmission events. We identified four examples with two or three isolates showing a remarkably low genetic distance, suggesting either direct transmission or a shared recent transmission event. Notably, in all four cases the respective isolates were collected at the same site, implying possible direct contacts. Unfortunately, no additional information on the contact networks or possible transmission risk factors are available for this cohort. Nevertheless, identification of such putative transmission events indicates that sequence analysis of HDV isolates can strongly contribute to a better understanding of the epidemiology (43) and may improve the characterization of the relevant infection routes (44). Moreover, when the putative transmissions can be placed in a temporal context, the analyses may allow conclusions about the incidence of HDV infections (45). We believe, the sequence in this study data support that recent transmission events still occur in Mongolia.

We provide evidence that the high HDV sequence diversity observed in Mongolia is at least partly caused by positive selection. Notably, the study does not directly address the extent of negative selection on the large HDAg. Negative selection refers to the process by which certain genetic traits are eliminated from a population because they confer a disadvantage to replication (46). As the Shannon entropy was not evenly distributed and some amino acids were highly conserved, negative selection appears to influence the genetic plasticity of HDV. This is in line with functional constrains of the multi-functional HDAg (47). In turn, our analysis with the HAMdetector tool indicates that HDV is under strong positive selection by HLA class I-associated selection pressure, which contributes to the HDV sequence diversity in Mongolia. Adaptation to $CD8^+$ T cell immune pressure by selection of escape mutations is well established for different viruses (48–50). Also, for HDV there is evidence supporting the selection of escape mutations from viral sequencing studies at the population level as well as from studies of individual $CD8^+$ T cell responses (15–17, 30, 51). The selected mutations typically interfere with the interaction of an infected cell with the T cell, either by impairing presentation of the variant epitope or by altering binding of the variant HLA/peptide-complex to the T cell receptor (48). The full epitope repertoire that is targeted in the L-HDAg in the context of different HLA-types is not well

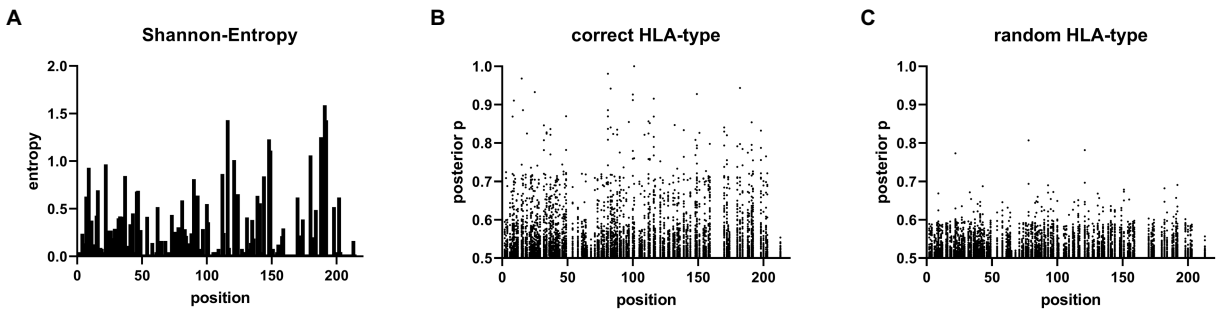

FIGURE 3
Sites under selection predicted by Bayesian regression model. **(A)** Shannon entropy map of the 131L-HDAg Sequences. **(B)** Posterior probability values >0.5 of the Bayesian regression model from the HAMdetector for each HLA-molecule on every amino acid in the L-HDAg are shown. **(C)** Posterior probability values of an HAMdetector run with the same alignment however with randomly assigned HLA-type.

TABLE 3 List of HAMs predicated on posterior probabilities.

HepMongolia				Habermann et al.			
Post.prob.	Allele	Position	Substitution	Substitution	Post. Prob.	Exp. confirmed	Comments
1	B*37	101	E	D101E	0.96	100-Q**D**HRRRKAL-109	Karimzadeh et al. (2019); see B*37 position 100
0.98075	A*68	81	I	V81I	0.97		
0.968	A*33	15	D	-	-		
0.9435	B*51	182	H	-	-		
0.942	A*33	83	A	-	-		
0.93275	B*48	25	R	-	-		
0.92775	C*07	149	T	-	-		
0.9265	B*37	100	E	-	-	100-**Q**DHRRRKAL-109	Karimzadeh et al. (2019); see B*37 position 101
0.9155	B*40	116	T	-	-		
0.912	A*01	100	E	-	-		
0.9105	A*33	9	K	-	-		
0.88575	B*54	81	I	-	-		
0.8855	A*33	16	I	-	-		
0.86975	B*54	49	L	-	-		
0.86925	A*33	81	I	-	-		
0.869	C*07	8	K	-	-		
0.86875	B*50	116	N	-	-		
0.8565	B*48	112	K	-	-		
0.85475	A*33	97	E	-	-		
0.854	B*58	191	G	-	-	189-RG**S**QGFPW-196	Kefalakes (2019)
0.852	B*08	113	N	K113N	0.96		
0.847	C*04	132	K	-	-		
0.846	B*13	32	K	-	-		
0.84075	C*04	170	D	-	-		
0.8405	A*33	83	T	-	-		
0.83675	B*58	81	I	-	-		

(Continued)

TABLE 3 (Continued)

HepMongolia				Habermann et al.			
Post.prob.	Allele	Position	Substitution	Substitution	Post. Prob.	Exp. confirmed	Comments
0.8365	C*07	37	T	-	-		
0.83475	A*33	88	K	-	-		
0.83375	B*27	139	G	-	-		
0.83225	B*54	198	L	-	-		
0.82975	B*15	170	D	S170N	0.99	170-SMQGVPESPF-179	Karimzadeh et al. (2019)
0.82775	A*31	34	T	-	-		
0.82625	B*13	151	D	-	-		
0.825	C*07	112	K	-	-		
0.82475	A*33	19	E	-	-		
0.8245	B*51	34	V	-	-		
0.8235	B*51	85	S	-	-		
0.822	B*51	88	G	-	-		
0.82125	A*03	37	T	-	-		
0.82125	B*40	109	Q	-	-		
0.82025	A*68	172	R	-	-		
0.816	B*08	116	R	-	-		
0.80875	B*13	81	V	-	-		
0.808	B*54	148	S	-	-		
0.80725	B*40	32	R	-	-		

defined yet and epitope mapping was just recently started (15, 17, 36, 37). Lack of experimental confirmation of novel epitope candidates is an important limitation of this study, however, isolation and storage of PBMCs for functional studies was not possible and would require a much larger effort and more advanced infrastructures. Nevertheless, the dataset provided here can support ongoing epitope mapping efforts by pointing toward regions under $CD8^+$ T cell selection pressure. A few residues with support for HLA class I-associated selection pressure were located inside described epitopes or overlapped with HLA-associated mutations from a previous study of a European cohort (17, 38). However, the majority of the associations were unique in the Mongolian cohort and did not overlap with associations in the European cohort. This highlights that viruses may differentially adapt in HLA-diverse populations as it has been suggested for HCV or HIV (52–54). Although this was not formally addressed in this study, such differential selection processes between populations may contribute to the formation of clusters or subtypes. Adaptation of HDV at the population level to common HLA molecules is an additional challenge for the development of T cell based immunotherapies, when escape variants are selected and accumulate.

Taken together, we provide a large dataset of HDV sequences from Mongolia in order to describe the extent of sequence diversity. Our data confirm previous studies showing that genotype 1 is the most frequent. Nonetheless, within this genotype the isolates from Mongolia belong to different clusters or to novel subtypes that have not yet been characterized. Phylogenetic analysis support that recent local transmission still occurs in Mongolia. One important driving factor for HDV sequence diversity is the adaptation to HLA class I-associated selection pressure.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: GenBank, OQ024240–OQ024371.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of the Mongolian Ministry of Health (study #2018-79-MEIC) and the Ethics Committee of the Medical Faculty of the Heinrich Heine University Düsseldorf (#2019-404-KFogU). The patients/participants provided their written informed consent to participate in this study.

Author contributions

This project was conceived by AW, BC and JT. Mongolian ethical permission, sample collection and sample logistic was coordinated by BC. Samples were collected by GE, AG, BM and BC. Experiments were performed by BM and AK. Data were analyzed by all authors.

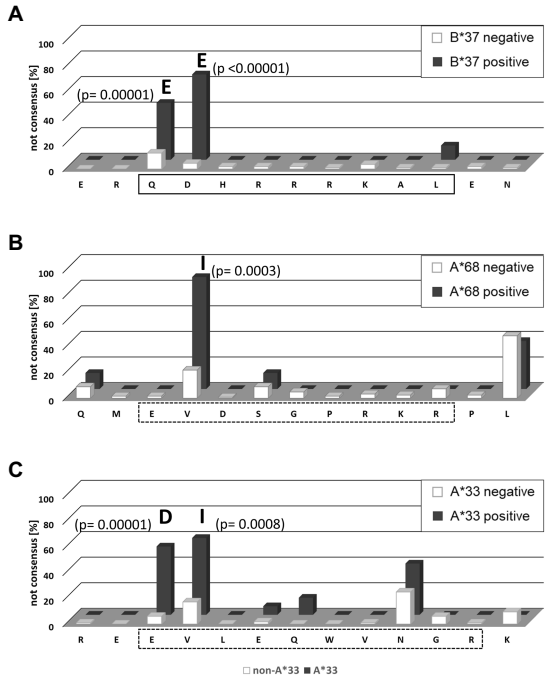

FIGURE 4
Frequency of HLA-associated viral polymorphisms in the epitope region predicted by the HAMDetector. The frequency of variations from the prototype sequence of the epitope region are shown for patients carrying the corresponding HLA-allele (black) and patients not carrying the corresponding HLA-allele (white). Positions with significant differences in polymorphism frequencies in the absence or presence of the corresponding HLA-molecule are marked, and *p* values (Fisher exact test) and the most frequent variant amino acid are indicated. **(A)** HLA-B*37 epitope region aa98–110. The confirmed epitope is outlined. **(B)** HLA-A*68 epitope region aa78–90. The predicted epitope is outlined in dashed lines. **(C)** HLA-A*33 epitope region aa13–25. The predicted epitope is outlined in dashed lines.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Distinct histological patterns in chronic hepatitis D with nucleos(t)ide analogue therapy

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Background: Chronic hepatitis delta virus (HDV) infection leads to a more severe hepatitis than hepatitis B virus (HBV) infection alone. Specific histological staining patterns have been described in HBV mono-infection, however this has not been extensively investigated in HDV co-infection. This study evaluated whether the use of nucleos(t)ide analogs (NAs) for concurrent HBV infection has an impact on the histological appearance of chronic HDV.

Methods: Liver biopsies of all patients referred for management of HDV infection were reviewed and hepatitis-specific stains for HBV antigens were evaluated. Clinical and histological characteristics were compared between patients on and off-NA therapy.

Results: 50 patients were included in our analysis, of which 26 (52%) were on NA therapy at the time of the biopsy. Overall, 8% stained for HBV core antigen and 86% stained for HBV surface antigen. On and off-NA groups had similar degrees of fibrosis and inflammation, however NA patients had an odds ratio of 7.15 for membranous staining and 0.13 for scattered granular staining (p =0.001). No association was found with markers of disease severity or viral activity, with nonetheless a lower score of total inflammation noted in biopsies with a positive membranous stain (8.5 vs. 10.3 p =0.04).

Conclusion: In chronic HDV infection, patients treated with nucleos(t)ide analogs demonstrate a unique membranous staining pattern for hepatitis B surface antigen, which is not associated with HBV or HDV replicative activity. These findings may help improve the understanding of the role of HBV directed therapy in HDV pathophysiology.

KEYWORDS

viral hepatitis, hepatitis D (delta) virus, nucleoside analog, histology, hepatitis B virus

Highlights

- Histological staining is associated with viral activity in chronic HBV, however this has been infrequently explored in HDV. In HDV, staining patterns differ based on HBV treatment status and do not appear to be associated with markers of viral activity.

Introduction

Chronic hepatitis delta virus (HDV) infection occurs only in patients with underlying hepatitis B infection (HBV) and leads to a more severe form of hepatitis, a more rapid progression to cirrhosis and an increased risk of hepatocellular carcinoma compared to HBV infection alone (1). Interferon-alfa based therapies, while not approved by the US Food and Drug Administration, are currently the only widely available treatments for HDV endorsed by major societies, but are plagued by low tolerability and sub-optimal results. The judicious use of nucleos(t)ide analogs (NA), pillars in the current treatment of HBV, has been integrated in practice guidance for HDV (2, 3). However NAs have been shown to be ineffective in controlling HDV viremia when used as mono-therapy and do not improve response to interferon (4, 5). Furthermore, the use of NAs in chronic HDV does not reduce the occurrence of hepatic decompensation, hepatocellular carcinoma, liver transplantation or death (6, 7).

Histologically, HDV is associated with a more severe liver injury and more active hepatitis than HBV infection alone (8). Specific histological staining patterns have not been extensively investigated in chronic HDV co-infection. In HBV mono-infection, HBV surface antigen (HBsAg) staining patterns correlate with the stage of disease and activity (9). Both nuclear HBV core antigen (HBcAg) expression and membranous HBsAg have been associated with viral replication (10–12).

In HDV, patterns of expression of hepatic HBV antigens are affected by viral interference and suppression of HBV replication. Overall, in co-infected patients, a smaller proportion of cases stain positive for HBV antigen stains (13, 14). Magnitude of HBsAg staining has also been associated with stages of liver disease in HDV (15). Hepatic expression of hepatitis delta antigen has been extensively described, correlating with the extent of inflammation on biopsy (16). However, while it is known that HDV and HBV antigens coexist in the liver (17), very few studies have evaluated the impact of HBV treatment on patterns of hepatic expression of HBV antigens in delta hepatitis. This study evaluated whether the use of NAs for concurrent HBV infection had an impact on the histological appearance of chronic HDV.

Materials and methods

Chronic HDV patients having undergone liver biopsy for evaluation and staging of liver disease at the National Institutes of Health between 2000 and 2020 were considered for analysis. Chronic HDV infection was determined by confirmation of the presence of HBsAg as well as anti-HDV antibodies in the serum and a positive delta antigen stain on liver histology and/or a quantifiable serum HDV RNA for more than 6 months.

Patients on treatment with Nucleos(t)ide analogs (Entecavir or Tenofovir) within the 6 months prior to the biopsy were considered as HBV on-treatment patients. All clinical characteristics were taken at the time of the biopsy, and laboratory test within 6 months from the biopsy were considered for analysis. All the data was collected from timepoints before the introduction of any additional HDV treatment with the exception of pegylated interferon. Recent interferon use was defined as within 6 months prior to biopsy. Patients treated in the context of clinical protocols were only included if biopsy was performed 6 months or more after withdrawal of HDV-directed therapy.

All biopsies were reviewed by an expert hepatopathologist (DEK) and biopsies performed both at the NIH Clinical Center and at outside centers were evaluated. Fibrosis stage was scored using Ishak fibrosis score and inflammation was assessed using the modified Histology Activity Index (HAI) on hematoxylin and eosin stain (18). All biopsies were processed in a similar fashion and hepatitis-specific stains for Hepatitis B surface antigen (HBsAg), Hepatitis B core Antigen (HBcAg) and Hepatitis D antigen (HDAg) were obtained. HBsAg (Thermo predilute, clone 3E7) and HBcAg (Dako B0586, 1:500) stains were performed on a Ventana Benchmark Ultra. HDAg stains were performed by manual staining using a high titer patient serum diluted at 1:1000 and detected using a biotinylated goat antihuman IgG (1:200, Vector Laboratories). Detection was performed using an avidin–biotin complex and diaminobenzidine chromagen (19). Four patterns of HBsAg staining were scored: inclusion-like, scattered granular, contiguous granular and membranous (all scored present or absent) (20). The HBsAg, HBcAg and HDAg stains were graded semi-quantitively on a scale of 0 to 3 (respectively 0%, <10%, 10–50 and > 50%) based on the proportion of the parenchymal staining. The overall HBsAg staining percentage was also noted, excluding the percentage of membranous staining which was reported separately. In addition to semi-quantitative grade for core-antigen, the proportion of nuclear and cytoplasmic proportion was estimated. Cases where HBsAg and HBcAg staining was inadequate were not considered in this analysis. Only initial biopsies were included in the overall analysis and follow-up liver biopsies (whenever available) were considered in a sub-analysis.

HDV RNA was measured using quantitative PCR with an assay from ARUP Laboratories, (Salt Lake City, UT, United States) (lower limit of detection 120 IU/mL), HBV DNA was detected using Roche COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, version 2.0 (lower limit of detection of 20 IU/mL) and quantitative HBsAg levels were measured with an assay from The Doctors Laboratory (TDL) (London, United Kingdom) (lower limit of detection 0.05 IU/mL).

Association between all clinical, laboratory and histological variables and staining patterns between the on and off-treatment groups was investigated. Correlation was evaluated using Spearman's rank correlation. Continuous variables were evaluated through *t*-tests or equivalent non-parametric tests (Wilcoxon rank sum test). Categorical variables were evaluated by Chi-squared or Fisher's exact test. Logistic regression was used to assess strength of association. p values <0.05 were considered significant. All data analysis was performed using R (Version 3.6.1).

Preparation and reporting of this manuscript adhered to the STROBE guidelines. All patients were enrolled in clinical research protocols conforming to the 1975 Declaration of Helsinki and approved by the National Institute of Diabetes and Digestive and Kidney Diseases Institutional Review Board and gave written informed consent for participation.

Results

Patient population

50 patients, 68% male with a mean age of 40.7 years at the time of the biopsy were included in our analysis. 24 (48%) were on NA therapy

at the time of the biopsy. While 21 patients had previously received additional HDV-directed treatment, only 7 patients received Interferon in the 6 months prior to the biopsy. 7 patients (14%) were HBe antigen (HBeAg) positive, while 39 (78%) were Anti-HBe Antibody positive (Figure 1) Mean HBV DNA was 19,600 international units (IU)/mL (Standard deviation (SD) 130,000), undetectable in 25 patients (50%), however with only two patients with an HBV DNA > 2000 IU/mL. Mean HDV RNA was 4.8 $\log_{10}$ IU/mL(SD 1.9) and was higher in the HBeAg positive subgroup (6.2 compared to 4.6 $\log_{10}$ IU/mL, $p = 0.012$). No differences in baseline serum HBsAb titers and HBV DNA were noted based on HBeAg status. Baseline mean ALT was 120 U/L, AST 84 U/L, total bilirubin 0.7 mg/dL and platelet count 158 $\times 10^9$/L. Additional baseline characteristics are described in Table 1.

In the overall study cohort, mean Ishak fibrosis score was 3.4 (SD 1.7), 14 patients (28%) had cirrhosis and mean total inflammation HAI score was 9.6 (SD 2.6). Overall, 8% of biopsies had a positive HBcAg stain and 86% expressed HBsAg. On average 30% of hepatocytes stained positively for HBsAg. In HBsAg positive samples, inclusion-like, scattered granular, contiguous granular and membranous patterns (Figure 2) were present in 77, 74, 72 and 40% of cases, respectively. In patients expressing a membranous pattern, an average of 75% of the hepatocytes stained in this pattern. In the 43 biopsies with available HDAg staining, 98% were positive.

Surface antigen staining associations with disease activity

There was no correlation between Hepatitis B surface antigen staining (in percentage) and markers of serum viral activity including serum HBsAg titers ($r = 0.29$, $p = 0.09$), HDV RNA ($r = 0.29$, $p = 0.07$), HBV DNA ($r = -0.17$, $p = 0.27$). Similarly, there was no correlation with serological markers of inflammation ALT ($r = -0.28$, $p = 0.08$), AST ($r = -0.23$, $p = 0.15$) or histological inflammation and HAI ($r = -0.22$, $p = 0.15$). Out of the four biopsies expressing HBcAg, overall staining was weak with nuclear expression in 0.25% and a cytoplasmic expression in 7.5% of hepatocytes.

Comparison between on and off-treatment groups

Baseline characteristics were comparable between both on and off-treatment groups (24 on-treatment and 26 off-treatment) and are presented in Table 1. There was no significant difference in previous treatment (either HBV treatment or interferon) as well as recent Interferon use. Liver enzymes were comparable between both groups although GGT levels were higher in the on-treatment group (83 vs. 63 U/L) ($p = 0.04$). Additionally, there was a higher prevalence of HBeAg positivity in the on-treatment group (25 vs. 3%) ($p = 0.045$). HBV and HDV viral loads, as well as histological scores of fibrosis and inflammation were not statistically different between groups.

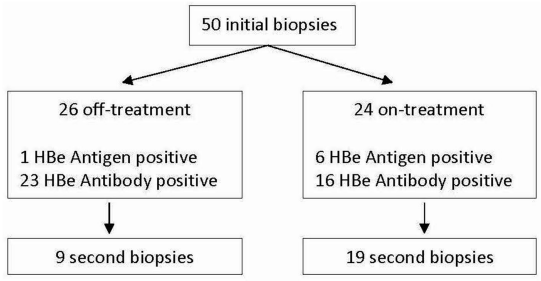

FIGURE 1
Study flow diagram.

Both on and off-NA groups had similar overall fraction of hepatocytes positive for HBsAg and HBcAg. However, patterns of HBsAg staining were different between on and off-NA patients and are presented in Table 2. A greater number of off-NA cases expressed a scattered granular pattern (59 vs. 91% $p = 0.014$) while a greater number of on-NA cases expressed a membranous pattern (59 vs. 19% $p = 0.007$). No difference was noted in the proportion of inclusion-like and contiguous staining patterns between the groups. With both staining patterns considered together, on-NA patients had an odds ratio of 7.15 for membranous staining and 0.13 for scattered granular staining (model χ^2 (2) =13.52, $p = 0.001$). This remained significant when controlled for recent Interferon use and HBeAg positivity, with an odds ratio of 12.03 for membranous staining and 0.03 for scattered granular staining in NA patients (model $\chi^2(4) = 28.26$, $p < 0.001$).

Associations with membranous staining pattern

No correlation was found between membranous staining intensity (by percentage) and the following markers of viral activity: HDV RNA ($r = 0.11$, $p = 0.68$), HBV DNA ($r = 0.31$, $p = 0.23$) and HBsAg levels ($r = 0.09$, $p = 0.73$). In addition, no correlation was noted with histological inflammation and fibrosis scores or the following biochemical markers: ALT, AST, ALP, GGT and platelets. When comparing cases with positive or negative membranous staining, there was no significant difference in serum levels of HDV ($p = 0.70$), HBV ($p = 0.96$), HBsAg ($p = 0.11$) and biochemical markers ALT ($p = 0.19$), AST ($p = 0.29$), ALP ($p = 0.63$), GGT ($p = 0.15$), total bilirubin ($p = 0.99$), and platelet counts ($p = 0.63$). However, while there was no difference in histological fibrosis ($p = 0.25$), a positive membranous stain was associated with a significantly lower HAI score (8.5 vs. 10.3 $p = 0.04$).

Associations with follow-up biopsy

In 28 patients, a follow-up liver biopsy performed on average 29 months after the initial biopsy (SD 32 months) was available. 68% of patients with a follow-up biopsy were treated with NAs at the time of the original biopsy. Overall, 89% stained positive for HBsAg and none for HBcAg. HBsAg staining remained stable over time in individual biopsies from first to second biopsy ($p = 0.22$) with an average of 24% of hepatocytes staining positive on follow-up biopsy.

Surface antigen staining patterns remained consistent over time, with the proportion of biopsies staining positive each pattern remaining identical; 88% had an inclusion-like pattern, 92% a scattered granular pattern, 72% a contiguous granular pattern and 68% a membranous pattern. Fisher's exact test non-significant for all four staining patterns. 8 patients (29%) expressed an identical staining pattern on repeat biopsy. Overall, magnitude of membranous staining

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TABLE 1 Baseline characteristics of the overall cohort and stratified by treatment status.

	Overall cohort (n =50)	Off treatment (n =26)	On treatment (n =24)	p value
Age (years)	40.7 (11.3)	41.7 (11.9)	39.56 (10.8)	0.50[c]
Race % (n)	Asian 52% (26)	Asian 42% (11)	Asian 63% (15)	0.29[a]
	White 40% (20)	White 50% (13)	White 29% (7)	
	Black/African-American 8% (4)	Black/African-American 8% (2)	Black/African-American 8% (2)	
Gender (Male) - %(n)	68% (34)	69% (18)	67% (16)	0.84[b]
Previous treatment - %(n)	42% (21)	42% (11)	42% (10)	0.96[b]
Recent Interferon treatment (<6 months) - %(n)	14% (7)	23% (6)	4% (1)	0.10[a]
Transient Elastography (kPa)	11.3 (7.0)	11.8 (8.0)	10.9 (6.5)	0.78[d]
Platelet count (x 10^9/L)	159 (61.7)	146 (66.8)	173 (53.7)	0.13[c]
GGT (U/L)	73 (73.3)	**83 (55.9)**	**63 (89.3)**	**0.03**[d]
ALP (U/L)	85(40.4)	86 (35.1)	85 (46.3)	0.73[d]
AST (IU/L)	84 (67.6)	82 (52.3)	86 (82.3)	0.49[d]
ALT (IU/L)	120 (129.0)	114 (97.7)	127 (158.3)	0.98[d]
Albumin (g/dL)	3.9 (0.5)	3.8 (0.4)	4.1 (0.5)	0.08[c]
Total bilirubin (mg/dL)	0.7 (0.5)	0.7 (0.2)	0.8 (0.7)	0.68[d]
PT (sec)	14.0 (1.0)	14.0 (1.2)	13.9 (0.8)	0.65[d]
HBV DNA (IU/mL)	19,600 (130400)	38,280 (182400)	57 (117)	0.26[d]
Undetectable serum HBV DNA-% (n)	50% (25)	48% (12)	52% (13)	0.57[b]
HDV RNA (IU/mL)	1,337,200 (2715200)	1,370,420 (3362480)	1,301,000 (1847160)	0.10[d]
Log HDV RNA (log_{10} IU/mL)	4.80 (1.90)	4.20 (2.30)	5.50 (1.00)	0.10[d]
Quantitative HBs Ag (IU/mL)	14,690 (14450)	13,750 (14720)	15,590 (14460)	0.59[d]
HbeAg positive %(n)	14% (7)	**4% (1)**	**25% (6)**	**0.045**[a]
HbeAb positive % (n)	78% (39)	88% (23)	67% (16)	0.06[b]
Ishak fibrosis	3.4 (1.7)	3.9 (1.6)	2.9 (1.7)	0.054[d]
HAI Score	9.6 (2.6)	9.8 (3.0)	9.5 (2.2)	0.63[c]
Hepatitis D Antigen stain	1.3 (0.6)	1.2 (0.4)	1.4 (0.7)	0.24[d]

Continuous variables are expressed as Mean (Standard deviation).
[a]Fisher's exact test.
[b]Chi squared.
[c]Independent t-test.
[d]Wilcoxon rank sum test.
Bold = p value < 0.05.

remained stable over time in the entire cohort (79% vs. 69%, p =0.7) and did not differ in individual biopsies (p =0.52). This stability in membranous staining over time was also seen in the sub-group already on NAs at the time of the first biopsy (p =0.81). In the sub-group of patients in which NA therapy was introduced in between both biopsies (n =7), 60% presented a scattered granular pattern with negative membranous staining, which was not seen in the follow-up biopsies. Furthermore, membranous staining was expressed in 67% of cases.

Discussion

In this retrospective analysis of 50 patients with chronic HDV infection, a unique HBsAg staining pattern differentiating patients on and off-treatment with nucleos(t)ide analogs for HBV is described. Overall, a minority of cases (8%) stain positive for HBcAg, however a majority of cases (86%) stain positive for HBsAg. While 40% of the study cohort expresses membranous HBsAg staining, a significant difference between patients on and off-NAs was observed with an odds ratio of 7.15 for membranous staining in treated patients. In addition, we demonstrate that HBsAg staining patterns remain stable on subsequent biopsies.

While it was initially thought that patients with delta hepatitis would exclusively stain for the delta antigen, additional studies described coexistence of both HBV antigens in chronic HDV patients (13, 17). Overall, in cohorts of HBV mono-infected patients, HBcAg is detected in 47–55% of hepatocytes, and HBsAg in 97% (with membranous expression in 41.5%) (21, 22). In HDV co-infection, intensity of HBsAg staining has been linked to earlier stages of disease and HBcAg positivity rates are low (15). The results from our cohort

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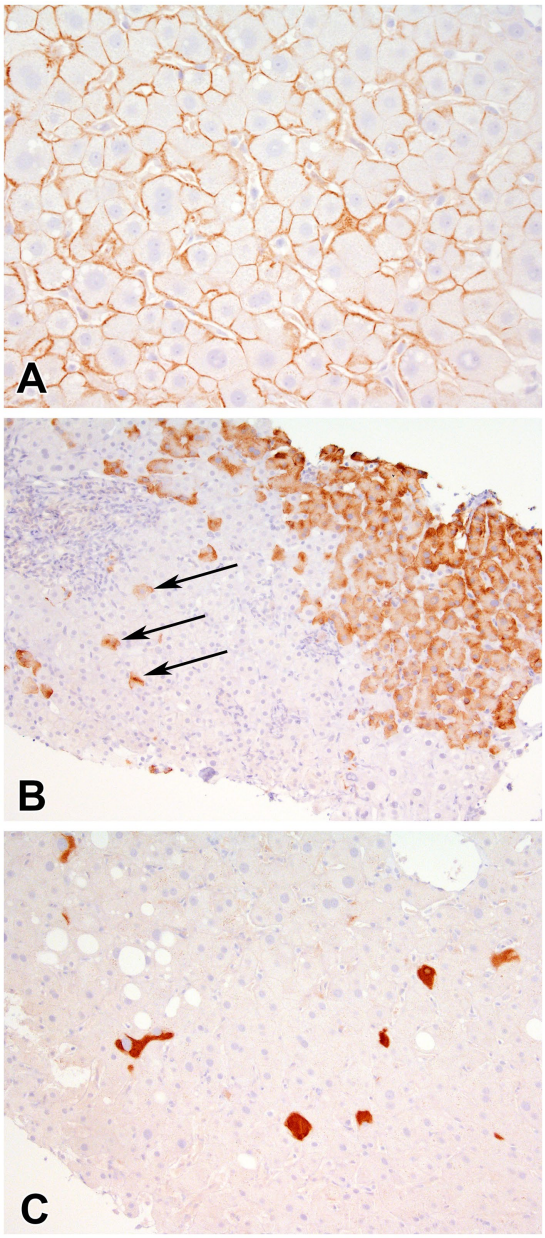

FIGURE 2
Immunohistochemical stain for Hepatitis B Surface antigen showing **(A)** membranous staining (400x), **(B)** scattered granular (arrows) and contiguous granular cytoplasmic staining (400X), and **(C)** inclusion-like (200X) staining of hepatocytes.

are consistent with previous reports of untreated chronic HDV patients, in which 6% of patients stained positive for HBcAg and 79.6% for HBsAg (14).

In our cohort, the low percentage of HBcAg staining is consistent with the low levels of HBV viral expression. Absence of HBcAg in the liver has previously been associated with low levels of HBV viral replication and minimal inflammatory activity (14, 23). A higher prevalence of HBcAg has been noted in the presence of higher viral loads in these cohorts (24). In the chronic HDV population, suppression of HBV replication is due to active HDV infection in addition to potential use of HBV therapy. In our cohort, the treated population includes non-cirrhotic patients who are either immune active patients or pre-core mutants; therefore a higher number of HBeAg positive cases in the on-NA group is expected. This also explains the lack of association with HBV viral activity markers in our subgroup of HBeAg positive patients.

In HBV, membranous HBsAg expression has been associated with active viral replication, and immune-mediated response (21, 25). However, other studies have not found a correlation between histological inflammatory activity and HBsAg staining intensity or pattern in HBV (26). In HDV co-infection, a previous study reported that membranous expression was seen less frequently in HDV positive patients than in HBV mono-infected patients (58% vs. 94% of cases) (27). The results from our study demonstrate a correlation between NA treatment and membranous staining without correlation to markers of HBV or HDV viral activity, a finding in line with previous descriptions of patterns of hepatitis delta antigen staining (16).

In addition to the findings regarding membranous staining, the overall prevalence of a scattered granular pattern in this cohort is reflective of persistent HBsAg positivity in all patients. In our cohort, no difference in serum quantitative hepatitis B surface antigen was noted based on NA status. Surface antigen levels remained elevated even in the context of suppressed HBV DNA. In a previous study, once stratified for histological severity of liver disease, no difference with serum titers of HBsAg was found between HBV mono-infected and HDV patients (14).

Patterns of staining in HDV patients in response to treatment have not been studied extensively. After interferon treatment in HBV, the HBsAg staining pattern has been shown to evolve from a cytoplasmic/inclusion pattern to a predominantly marginal/negative pattern (28). In a HBV/HDV co-infected cohort, a decrease in intensity of HBsAg and HBcAg was noted at the conclusion of interferon therapy (15). No differences in HBsAg and HBcAg expression in the liver were noted in a subgroup of patients after adefovir treatment (15). Our study adds to the body of evidence regarding hepatic expression of HBV antigens in chronic HDV infection and describes a pattern of staining associated with NA use. Furthermore, this association remains significant even when recent interferon use is taken into account. This finding could be reflective of the diversion of the HBV surface antigen toward HDV assembly in cases where the HBV polymerase is inhibited. This particular staining pattern can represent aborted viral replication, residual production from integrated HBV DNA and persistence of covalently closed circular DNA, stages in the infectious cycle which are not targeted by NAs (29).

This study is not without limitations. Due to the relative rarity of chronic HDV in most clinical settings as well as the heterogeneous presentation of liver disease in this population, a controlled comparison with HBV mono-infected patients and stratified on severity of liver disease, viral activity and treatment status was not feasible. In addition, histopathological assessment of viral hepatitis can potentially be influenced by sampling error and the sensitivity of assays. In addition, due to the retrospective nature of this cohort, assessment of serum and intrahepatic viral activity through novel assays could not be performed. Furthermore, impact of duration of NA therapy could not be assessed, as therapy was often initiated before referral to our center. Nonetheless, our sizeable real-world

TABLE 2 Hepatitis B histological staining patterns compared between On and Off treatment groups.

	Overall cohort	Off treatment	On treatment	*p* value
	(*n* =50)	(*n* =26)	(*n* =24)	
Surface Antigen positive % (*n*)	86% (43)	81% (21)	92% (22)	0.19[c]
Surface Antigen score (mean-SD)	1.6 (1.0)	1.4 (0.9)	1.8 (1.1)	0.11[b]
Score 0	7	5	2	
Score 1	20	9	11	
Score 2	11	10	1	
Score 3	12	2	10	
Surface Antigen % hepatocytes (mean-SD)	30% (32)	20% (18)	40% (38)	0.21[b]
Inclusion positive % (*n*)	77% (33)	67% (14)	86% (19)	0.16[c]
Scattered granular positive % (*n*)	74% (32)	**91% (19)**	**59% (13)**	**0.02**[a]
Contiguous granular positive % (n)	72% (31)	62% (13)	82% (18)	0.15[a]
Membranous positive % (*n*)	40% (17)	**19% (4)**	**59% (13)**	**0.007**[a]
Membranous % hepatocytes (mean-SD)	75% (29)	63% (3)	79% (26)	0.35[b]
Core Antigen score (mean-SD)	0.1 (0.4)	0.2 (0.5)	0.04 (0.2)	0.36[b]

SD: Standard deviation.
[a]Chi squared.
[b]Wilcoxon rank sum.
[c]Fishers exact test.
Bold = p value < 0.05.

cohort benefits from an extensive virological assessment and thorough histological analysis. Our results highlight that the histopathological evaluation of HDV cannot be interpreted solely like HBV and that treatment can modulate histological expression.

In conclusion, use of HBV nucleo(s)tide analog therapy in chronic HDV infected patients resulted in a unique Hepatitis B surface antigen membranous staining pattern. No association between serological markers of disease activity and membranous staining pattern was found in this HDV cohort, which differs from previous reports in HBV mono-infection. The use of HBV suppressive therapy may have allowed for indirect evidence of diversion of HBV surface antigen toward HDV replication, a finding not previously seen histologically. Further understanding of the role of nucleos(t)ide analog therapy in HDV may allow for an improved understanding of the pathophysiology of HDV infection.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation upon written request.

Ethics statement

The studies involving human participants were reviewed and approved by National Institutes of Health Institutional Review Board (IRB). The patients/participants provided their written informed consent to participate in this study

Author contributions

JH, DEK, and CK: study conception and design. JH, JG, DEK, and CK: acquisition of data. JH, TH, JG, and DEK: analysis and interpretation of data. JH, DEK, and CK: drafting of the manuscript. JH, TH, JG, DEK, and CK: critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Non-organ-specific autoantibodies with unspecific patterns are a frequent para-infectious feature of chronic hepatitis D

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Infections with hepatotropic viruses are associated with various immune phenomena. Hepatitis D virus (HDV) causes the most severe form of viral hepatitis. However, few recent data are available on non-disease-specific and non-organ-specific antibody (NOSA) titers and immunoglobulin G (IgG) levels in chronic hepatitis D (CHD) patients. Here, we examined the NOSA titers and IgG levels of 40 patients with CHD and different disease courses and compared them to 70 patients with chronic hepatitis B (CHB) infection. 43% of CHD patients had previously undergone treatment with pegylated interferon-α (IFN-α). The antibody display of 46 untreated patients diagnosed with autoimmune hepatitis (AIH) was used as a reference. The frequency of elevated NOSA titers (CHD 69% vs. CHB 43%, $p<0.01$), and the median IgG levels (CHD 16.9g/L vs. CHB 12.7g/L, $p<0.01$) were significantly higher in CHD patients than in patients with CHB, and highest in patients with AIH (96%, 19.5 g/L). Also, the antinuclear antibody pattern was homogeneous in many patients with AIH and unspecific in patients with viral hepatitis. Additionally, f-actin autoantibodies were only detectable in patients with AIH (39% of SMA). In CHD patients, IgG levels correlated with higher HDV viral loads, transaminases, and liver stiffness values. IgG levels and NOSA were similar in CHD patients irrespective of a previous IFN-α treatment. In summary, autoantibodies with an unspecific pattern are frequently detected in CHD patients with unclear clinical relevance.

KEYWORDS

hepatitis D virus, hepatitis B virus, autoimmune hepatitis, autoantibodies, viral hepatitis

1. Introduction

Hepatitis D virus (HDV) infection is the most severe form of viral hepatitis. HDV is a defective RNA virus and requires the hepatitis B surface antigen (HBsAg) of the hepatitis B virus (HBV) to complete its lifecycle. Worldwide, 15–20 million people suffer from a chronic HDV infection, which amounts to about 6–8% of the patients with a chronic HBV infection (CHB) (1). Chronic HDV/HBV co- or super-infection (CHD) is associated with higher rates of liver cirrhosis, hepatocellular carcinoma, and higher liver-related mortality compared to patients with HBV mono-infection (2–5). Chronic HDV infection has been a challenge in clinical hepatology in terms of diagnostics, monitoring, and therapy. For example, anti-HDV Immunoglobulin M (IgM) testing was used to diagnose ongoing hepatitis delta replication for a long time before HDV-RNA assays became available, and there have been incremental advances in the standardization of these molecular assays (6, 7). Antiviral treatment with pegylated Interferon-α (IFN-α) has been introduced around 10 years ago with low treatment response rates (8), and only lately therapy with bulevirtide received conditional approval in the EU (9).

Autoimmune hepatitis (AIH) is an immune-mediated chronic liver disease leading to (necro)inflammation, liver cirrhosis with all of its complications. AIH is characterized by elevated serum transaminases and immunoglobulin G (IgG) levels, detectable autoantibodies, and characteristic histopathological findings. Based on different autoantibody profiles, AIH can be classified into two subtypes, autoimmune hepatitis type 1 (AIH-1) and type 2 (AIH-2). (10, 11) Over the years there have been attempts to standardize the assessment of autoantibody profiles (12).

Notably, patients with chronic viral hepatitis may also develop high autoantibody titers (13, 14). Furthermore, overt extra-hepatic manifestations, such as vasculitis, polyarthritis nodosa, glomerulonephritis, dermatitis, polyarthralgia, arthritis, lung disease, and aplastic anemia may also occur (13, 15). Molecular mimicry and bystander activation have been proposed as possible mechanisms to explain the breakdown of self-tolerance caused by viral infection (16). Differentiating autoimmune phenomena in viral hepatitis from true autoimmune (liver) disease is of utmost clinical relevance: a concomitantly existing autoimmune liver disease would necessitate immunosuppressive treatment, which bears the risk of dampening control of the virus-induced liver disease.

Most of the antibodies in viral hepatitis are non-disease-specific and non-organ-specific antibodies (NOSA). Antinuclear antibodies (ANAs) were the first autoantibodies to be associated with AIH (17). Since NOSA have also been detected in patients with viral hepatitis, drug-induced liver injury, Wilson's disease, alcohol-induced liver disease, non-alcoholic fatty liver disease, and a variety of extrahepatic autoimmune diseases, the specificity of ANAs for a specific (liver) disease is generally low (18).

Anti-smooth muscle antibodies (SMAs) are detected in up to 85% of patients with AIH-1 (19). The SMA titer is also of clinical significance since higher titers have higher AIH-specificity (11). Moreover, sub-specificity toward f-actin has been clearly associated with AIH (11). However, SMA can also be observed in up to 25% of patients with CHB, and chronic hepatitis C virus (HCV) infection (19–22).

The anti-liver-kidney microsomal type 1 antibodies (LKM1), which are targeted against cytochrome P450 CYP2D6 (23) are a hallmark in the diagnosis of AIH-2. In 1983, LKM autoantibodies were also described in CHD patients and later termed LKM-3 (24). These autoantibodies were present in 13–14% of Italian HDV carriers. The major LKM-3 autoantigen was identified as an epitope on family 1 UGTs (UGTl) (25).

Anti-mitochondrial antibodies (AMA) are typically present in patients suffering from primary biliary cholangitis (18) but have also been detected in a small number (3%) of patients with HCV (26). Anti-soluble liver antigen/liver-pancreas antibodies (SLA/LP) are the most specific for AIH-1 among all AIH-related antibodies. However, only a small proportion of patients with AIH show these antibodies that are associated with persistent disease (18, 27, 28).

In patients with CHD, basal cell layer (BCLA) and antithymic antibodies were frequently found, too (29).

The first evidence for autoantibodies in CHD was established as early as 1980 (30). However, in comparison to HBV and HCV infection (31, 32), data for autoimmune phenomena in chronic HDV infection is scarce (31). At the time when the last results on this topic were published nearly 30 years ago, antibody assessment was less standardized, and neither HDV PCR diagnostics, transient liver elastography, nor HDV interferon treatment was readily available (6, 7, 9).

A better knowledge of the autoantibody profiles of CHD might contribute to the understanding of immune-mediated disease progression and extrahepatic manifestations. Autoantibodies could also serve as suitable biomarkers to support the assessment of disease activity, to predict the clinical course, and treatment outcome.

In this study, we cross-sectionally evaluated the autoantibody titers and IgG levels in a single-center cohort of patients with CHD and different disease statuses and compared those to patients with CHB mono-infection and AIH. NOSA titers and IgG levels were correlated to HDV viremia, transaminases, and liver stiffness values from transient elastography as possible markers for the clinical status.

2. Materials and methods

2.1. Study population

We retrospectively identified and analyzed three different cohorts consisting of 46 patients with AIH, 42 patients with CHD, and 70 patients with CHB. Autoantibody titers were available in 46/46 patients of the AIH cohort, in 40/42 patients of the CHD cohort, and 69/70 patients of the HBV cohort. IgG levels were available in 44/46 patients of the AIH cohort, in 36/42 patients of the CHD cohort, and 61/70 patients of the HBV cohort.

All patients were treated in specialized outpatient clinics at the University Medical Center Hamburg-Eppendorf. The data were collected as part of the clinical routine visits between 2010 and 2019 and retrospectively analyzed. The diagnosis of AIH was secured by serological and histopathological findings as well as treatment response according to the EASL clinical practice guidelines (33). Viral hepatitis was excluded in patients of the AIH group. Inclusion criteria for participants in the CHD cohort were the confirmation of positive HBsAg status as well as proof of anti-HDV, not necessarily an existing viremia. In this present study, 19/41 (46%) of patients with HDV infection had a negative HDV PCR at the time of inclusion.

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Patients receiving INF-α therapy within the last 6 months before inclusion, with a history of malignancy, HCV infection, or HIV were excluded. The study was approved by the local ethics committee (WF-035/17).

2.2. Antibody diagnostics

Testing for autoantibodies was performed by trained personnel at the Institute of Immunology at the University Medical Center Hamburg-Eppendorf. ANA, SMA, LKM-1, and AMA were assessed by immunofluorescence testing. HEp-2 Cells (human epithelioma cells) and tissue slides (Euroimmun, Lübeck, Germany) were incubated in accordance with the manufacturer's instructions, and interpretation was performed manually using the Eurostar microscope (Euroimmun, Lübeck Germany). SLA was tested by ELISA using enzyme immunoassays from Euroimmun, Germany (11, 34). According to our internal laboratory criteria, we regarded ANA, SMA, and LKM-1 as positive at a titer of >1:80, AMA as positive at titers of ≥1:40, and SLA/LP as positive if ≥20 U/mL. IgG levels were deemed elevated above the cut-off 16 g/L.

2.3. Statistics

All data were collected in a Microsoft® Excel® (Version 12.3.6 for Mac) file and imported into IBM *SPSS* (Version 24.0 for Mac, Armonk, NY, United States, 2016) for statistical analysis.

Data are presented as absolute numbers and percentages or as the median and interquartile range (IQR). Nominal variables were compared using the Chi-Square test. Metric variables were analyzed using the Mann–Whitney-U-test because there was no normal distribution. Spearman's rho was used for correlation analysis: the statistical significance is indicated following the correlation coefficient (r) (*$p < 0.05$, **$p < 0.01$).

3. Results

3.1. Baseline characteristics of the study population

The baseline characteristics of the three patient cohorts are summarized in Table 1. The three cohorts differed in terms of age: patients in the CHB cohort had a median age of 37 years, in the CHD cohort the median age was 45 years, and 54 years in the AIH cohort. Not surprisingly, there were significantly more men in the HDV cohort than in the AIH cohort since AIH is known to be a disease with predominance in women (60% versus 33%) (10).

In the CHB cohort, 16% were under treatment with nucleoside/nucleotide analogs (NA) and had a median viral load under the limit of detection (0–456 IU/mL); 1% had previously undergone treatment with IFN-α and had an HBV DNA load of 130 IU/mL; 83% were therapy naïve and had a median viral load of 2,400 (422–20,250) IU/ml.

In the CHD cohort, 17% of patients were under treatment with nucleoside/nucleotide analogs (NA) and had median HDV and HBV viremia underneath the detection limit (HDV RNA: 0–12,200 IU/mL; HBV DNA: 0–200 IU/mL); 43% of HDV patients had previously undergone treatment with IFN-α and had a median HDV viremia of 32,000 IU/mL (870–220,000) and a median HBV viremia of 70 (6–790) IU/ml; 40% were therapy naïve and had a median HDV viremia under the limit of detection (0–300 IU/mL) and a median HBV DNA load of 378 (35–3,490) IU/ml.

TABLE 1 Baseline characteristics of the study population.

	AIH (n=46)	CHD (n=42)	CHB (n=70)
Age (years)	54 (37–71)	45 (37–54)	37 (30–47)
Sex (female: male)	31:15	17:25	40:30
ASAT (U/l)	202 (68–801)	40 (26–92)	22 (18–31)
ALAT (U/l)	278 (76–758)	60 (32–117)	31 (19–47)
Viral load (U/ml) in patients currently treated with NAs	–	n = 7 HDV: 0 (0–12,200) HBV: 0 (0–200)	n = 11 0 (0–456)
Viral load (U/ml) in patients with a history of IFN-α treatment	–	n = 17 HDV: 32000 (870–220,000) HBV: 70 (6–790)	n = 58 2,400 (422–20,250)
Viral load (U/ml) in therapy naïve patients	–	n = 17 HDV: 0 (0–300) HBV: 378 (35–3,490)	n = 1 130
Transient elastography (kPA)	n = 27 8.2 (6.1–17.9)	n = 36 7.7 (6.2–14.0)	n = 40 5.3 (4.3–6.5)

Values are medians with an interquartile range (IQR).

82% of AIH patients were treatment naïve at the time of study inclusion. 7% of patients had received steroids (prednisolone or budesonide) before, and another 11% had previously been treated with steroids and azathioprine.

3.2. IgG levels in patients with viral hepatitis compared to AIH patients

Patients with CHD had statistically higher IgG levels than patients with CHB ($p < 0.01$), but lower IgG levels than patients with AIH ($p < 0.05$). In the AIH cohort, the median IgG level was 19.5 g/L (15.5–27.4) and was elevated in 73% of cases. In the CHD cohort, IgG levels were measured with a median value of 16.9 g/L (12.3–22.4). Elevated IgG levels were detected in 54% of CHD patients. In the CHB cohort, the median IgG level was 12.7 g/L (10.2–14.3) and was elevated in 12% (Figure 1). In addition, patients with CHD showed a weak correlation between IgG levels and HDV viral load levels ($r = 0.39$*), whereas there was no correlation between IgG levels and HBV viral loads. Thus, patients with detectable HDV RNA (46%) had significantly higher IgG levels compared to patients with undetectable HDV RNA ($p > 0.01$). Patients with undetectable HDV RNA had similar IgG levels to patients of the CHB cohort. IgG levels were similar in viremic CHD patients regardless of the IFN-α treatment status. IgG levels also

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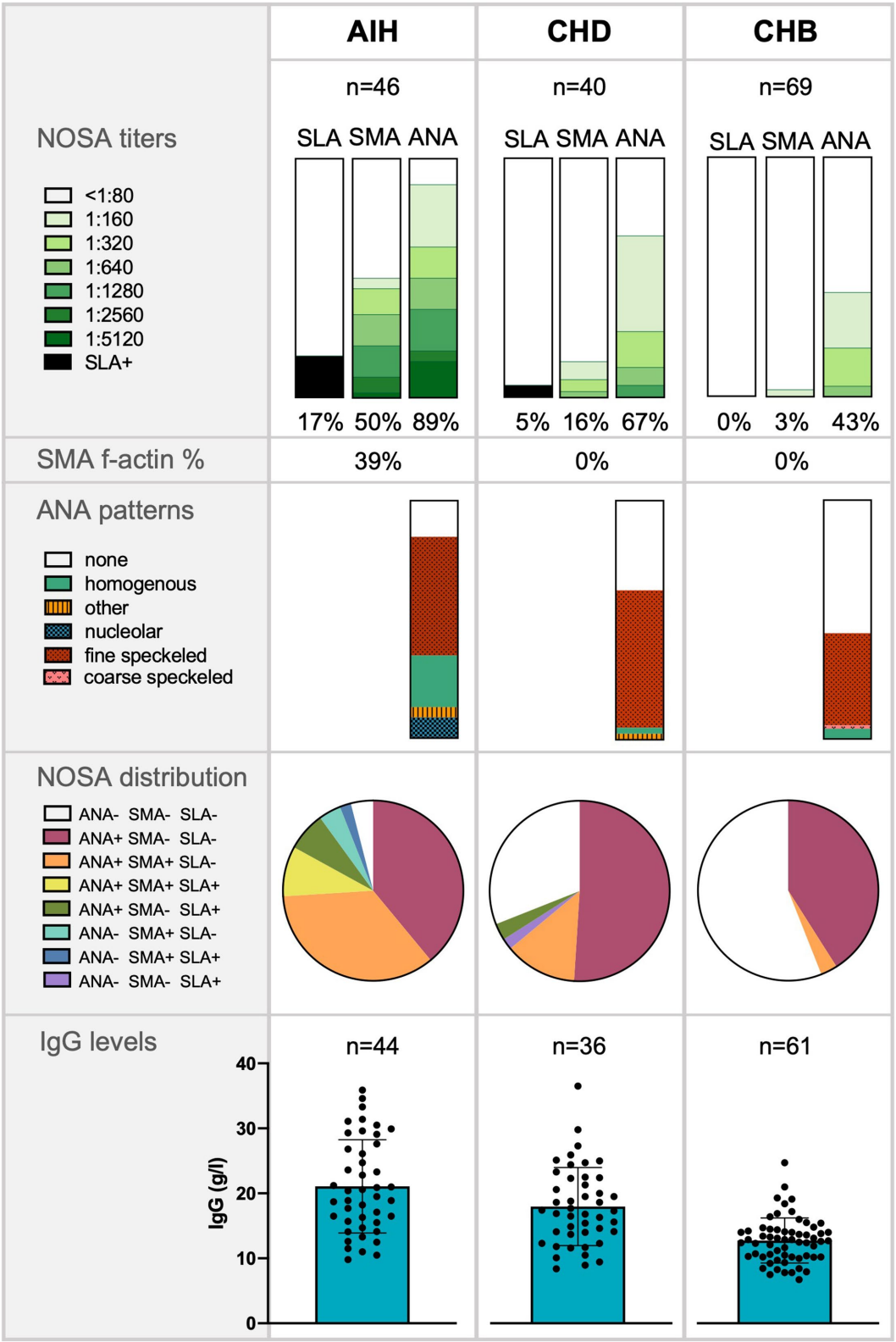

FIGURE 1
Frequency of non-disease-specific and non-organ-specific antibodies (NOSA), antinuclear antibodies (ANA) patterns, NOSA distribution, and Immunoglobulin G (IgG) levels in patients with autoimmune hepatitis (AIH), chronic hepatitis D (CHD), and chronic hepatitis B (CHB). The significance of IgG levels was tested with a Mann–Whitney-U test: AIH compared to CHD ($p<0.05$*); CHD compared to CHB ($p<0.01$**).

TABLE 2 Spearman correlation analysis of ANA and SMA titers as well of IgG levels with laboratory parameters in all cohorts.

ANA titers with	AIH (n=46)	CHD (n=40)	CHB (n=70)
ALAT	−0.144	0.118	−0.122
ASAT	−0.156	0.361*	−0.096
IgG level	0.071 (n = 44)	0.419 (n = 36)*	−0.087 (n = 61)
HBV viral load	–	−0.107	0.079
HDV viral load	–	−0.131	–
Age	0.285	−0.032	−0.093
Liver stiffness	−0.029	0.218	−0.037
SMA titers with			
ALAT	0.187	0.133	−0.041
ASAT	0.183	0.182	0.063
IgG level	0.342 (n = 44)*	0.098 (n = 36)	−0.067 (n = 61)
HBV viral load	–	−0.183	0.100
HDV viral load	–	0.165	–
Age	−0.202	−0.188	−0.085
Liver stiffness	0.002	0.526**	0.033
IgG level with	**AIH (n =44)**	**CHD (n =36)**	**CHB (n =61)**
ALAT	0.217	0.510**	0.064
ASAT	0.330*	0.771**	−0.022
HBV viral load	–	−0.158	0.160
HDV viral load	–	0.389*	–
Age	−0.029	−0.209	−0.160
Liver stiffness	0.192	0.410*	0.364*

*p < 0.05, **p < 0.01.

correlated weakly significantly with ANA titers in patients with CHD (r = 0.42*). In patients with AIH, IgG levels correlated weakly with SMA titers (r = 0.34*; Table 2).

3.3. Levels, staining patterns, and distribution of non-organ-specific antibodies in patients with viral hepatitis compared to AIH

Positive NOSA titers were found more frequently in the CHD cohort than in the CHB cohort (CHD 69% vs. CHB 43%, p < 0.01). However, positive NOSA titers were significantly less frequent in patients with HDV infection than in patients diagnosed with AIH (CHD 69% vs. AIH 96%, p < 0.01). Notably, the patterns differed between the cohorts: AIH patients displayed significantly more homogenous ANA autoantibodies than the other cohorts. In addition, AIH patients displayed significantly more f-actin autoantibodies than the other cohorts. Details of the ANA staining patterns are shown in Figure 1.

Positive ANA titers were less frequently detected in patients with CHD than in patients with AIH (CHD 67% vs. AIH 89%, p < 0.05). In addition, significantly fewer patients with HDV had high ANA titers of ≥1:320 than patients with AIH (CHD 28% vs. AIH 63%, p < 0.01). However, patients with HDV infection were more likely to have positive ANA titers than patients with CHB (CHD 67% vs. CHB 43%, p < 0.05). CHD patients with detectable HDV RNA were not more likely to have positive ANA titers or ANA titers of ≥1:320 compared to CHD patients without detectable HDV RNA.

In general, positive SMA titers were most frequently observed in patients with AIH (AIH 50% vs. CHD 16% vs. CHB 3%). Specifically, 91% of patients with positive SMA titers in the AIH cohort had high SMA titers of ≥1:320. Patients with CHD tended to have positive SMA titers more frequently than patients with CHB (CHD 16% vs. CHB 3%, p = 0.055). In addition, detectable HDV RNA tended to be associated with positive and high SMA titers ≥1:320 compared to patients with undetectable HDV RNA (>1:80: p = 0.08; ≥1:320: p = 0.08).

Elevated SLA titers were detected in one patient (3%) with CHD and eight patients (17%) diagnosed with AIH, but not in any patient with CHB.

Neither patients with viral hepatitis nor patients with AIH had positive AMA or LKM-1 titers.

Among CHD patients, ANA titers were higher in IFN-α naïve patients than in those who were treated with IFN-α at some time before (median 1:1280 vs. 1:160). SMA titers were detected independently of previous IFN-α treatment. Among the CHD patients who received IFN-α therapy 3/18 (17%) showed sustained response during follow-up. For these patients, NOSA titers were determined before and after treatment and remained unchanged.

The distribution of NOSA was analyzed for each cohort and visualized in Figure 1: Patients with CHB almost exclusively had elevated ANA titers with negative SMA and negative SLA titers. In patients with CHD and patients with AIH, positive ANA titers were also combined with positive SMA titers. However, this pattern occurred significantly less frequently in patients with CHD than in patients with AIH (CHD 13% vs. AIH 35%, p < 0.05). The CHD patient with positive SLA titer was negative for SMA and ANA.

NOSA titers did not correlate with HDV viral load levels.

3.4. Antibodies and clinical parameters

Transaminases, alanine-aminotransferase (ALAT) and aspartate-aminotransferase (ASAT) were significantly higher in the AIH cohort compared to the two viral hepatitis cohorts (p<0.01). However, transaminases were significantly higher in patients with HDV infection compared to patients with CHB (p<0.01). A moderate or weak correlation between transaminases (ALAT; ASAT) and IgG levels were observed in patients with CHD (r=0.51**; r=0.77**) and AIH (r=0.22; r=0.33*). Transaminase levels were significantly higher in patients with viremic HDV infection than in patients with undetectable HDV RNA, in whom transaminase levels did not exceed those of patients of the CHB cohort. Moreover, a weakly significant correlation between ANA titers and ASAT was seen in patients with HDV infection (r=0.36*).

There was no statistical difference in liver stiffness values determined by transient elastography among the three different patient cohorts (in kPa: CHB 5.3 (4.3–6.5); CHD 7.7 (6.2–14.0); AIH 8.2 (6.1–17.9)). In CHD patients, liver stiffness values did not correlate with ANA titers (r = 0.22), but showed a moderate correlation with

SMA titers ($r = 0.53^{**}$). In the CHD cohort, liver stiffness values also correlated with HDV viral load ($r = 0.52^{**}$) and IgG levels ($r = 0.41^{*}$). CHD patients with liver cirrhosis (liver stiffness values >14 kPa) had significantly higher IgG levels compared to CHD patients with low liver stiffness values (< 6.5 kPa) ($p < 0.05$). Also, in CHB patients, IgG levels weakly correlated with liver stiffness values ($r = 0.36^{*}$). In the AIH cohort, no correlation was found between liver stiffness values and ANA titers, SMA titers, or IgG levels.

3.5. Histopathological findings of all cohorts

Biopsies were available for 34 AIH patients, 12 patients with CHD, and 3 patients with CHB (Supplementary Tables 1–3). The median Desmet score for liver fibrosis/cirrhosis was similar for AIH and CHD patients (2/4). The biopsies of patients with CHB did not show fibrosis/cirrhosis (0/4). The median modified Ishak score (mHAI) was higher in AIH patients (9/18, IQR 4–16) compared to patients with CHD (6.5/18, IQR 2–10) and CHB (4,5/18, IQR 3–6), without reaching statistical significance.

4. Discussion

Infections with hepatotropic viruses have been associated with various immunopathological manifestations. An association between infection and autoimmunity is well documented, particularly for chronic infections with hepatitis B and hepatitis C viruses (31). In comparison, less data was available on the frequency and pattern of NOSA for CHD and in relation to the disease or treatment status (30, 35).

In chronically HDV-infected individuals, perforin-positive cytotoxic CD4+ T cells accumulate and have been implicated in contributing to the severity of HDV related liver infection (36–38). Also, HDV infection was shown to augment the antiviral state of the hepatocytes, chemokine production, and antigen presentation (39, 40). Though the liver damage that results from chronic HDV infection is considered to be primarily mediated by the immune system (37, 41), little is known about the role of autoantibodies.

In this current study, we cross-sectionally examined available NOSA titers and IgG levels in 42 patients with CHD and 116 controls consisting of 70 patients with CHB, and 46 patients diagnosed with AIH. Because patients with CHD were compared to patients with HBV mono-infection, differences in autoimmune phenomena, represented by autoantibody titers and IgG levels, could be associated with the addition of HDV infection.

This study is limited by its retrospective design, the absence of multicenter validation, and the small sample size. Also, the three cohorts were not matched for age, sex, or degree of liver damage. Only very selected autoantibodies were analyzed as used in the standard workup of elevated liver enzymes. This panel is primarily matched to the autoantibody profile of AIH, PSC, and PBC.

Here, almost exclusively patients with HDV genotype 1 infection were included, as this is the most common genotype circulating in Europe (42). However, recent studies have shown differences in spreading kinetics, treatment outcome, and disease courses between variant HDV genotypes (43, 44). Therefore, it would be of interest whether the autoantibody profile differs, too.

Elevated serum IgG levels are considered a characteristic feature of AIH, as they are detected in up to 85% of patients and are part of the diagnostic score in AIH (45). We found elevated IgG levels in 73% of patients with AIH, and only in 12% of patients with CHB, but also in 54% of patients with CHD. Hartl et al. previously suggested a correlation between ANA/SMA titers and IgG levels in patients with AIH, but IgG levels were not related to the degree of liver fibrosis or intrahepatic inflammatory activity (45). In this current study, there was also a significant correlation between IgG and ANA titers in CHD patients and SMA and IgG in AIH patients. However, there was also a significant correlation between IgG and transaminases, suggesting a relationship between IgG and intrahepatic inflammatory activity in CHD and AIH patients. Also, in the CHD cohort IgG levels and HDV viral loads correlated with higher liver stiffness values. Patients with HDV viremia had significantly higher IgG levels than patients without detectable viremia.

Most data available on the frequency of autoantibodies in CHD patients is historic, when the methodologies applied differend, were less standardized, and IFN-α treatment was not available. In this study, patients with CHD were significantly more likely to have positive/high NOSA titers than patients with CHB (CHD 69% vs. CHB 43%; $p < 0.01$), but less likely to have positive NOSA titers than patients with AIH (CHD 69% vs. AIH 96%; $p < 0.01$). The prevalence of positive NOSA titers in AIH patients determined in our study was consistent with the literature (46). In a study by McFarlane et al. from 1995, about 20% of CHD patients ($n = 27$) had positive NOSA titers, similar to the included patients with HBV mono-infection ($n = 22$) (35). In a study with 325 Chinese patients diagnosed with CHB, 58.2% showed positive NOSA titers at the same cut-off value as in our analysis, which was significantly higher than in healthy controls (6.7%) (47).

In line with our results, ANA were among the most frequent NOSA in the CHB patients of the Chinese cohort (47). The data base for ANA in CHD is scarce. In contrast to our results, in 1986, Zauli et al. found the prevalence of ANA to be significantly lower in CHD patients (9%) than in CHB patients (48).

Patients with AIH were significantly more likely to have positive SMA titers and, in particular, to have high titers (≥ 1:320) than patients with CHD or CHB. However, 8% of patients with CHD also showed SMA titers, while these antibodies were not present in CHB patients. While patients with HBV mainly had isolated elevated ANA titers, patients with HDV and AIH also had combined elevated ANA and SMA titers. In a previous study from 1986, the prevalence of SMA in CHD did not significantly differ from chronic hepatitis HBV mono-infection (48). Here, SMA titers correlated with liver stiffness values in CHD patients but not in the other cohorts. Larger studies should be conducted to validate whether high SMA titers are indeed associated with activity or prognosis of the HDV liver disease.

SLA is considered the most specific marker for AIH among all AIH-related antibodies. It is still controversial whether the detection of SLA is associated with severe courses of AIH (18, 27).

Rarely, do HCV/LKM-1 positive patients progress to LKM-1 positive autoimmune hepatitis (49). Positive LKM-1 was not detected in any patients of this current study. Whereas the presence of LKM-1 is a typical feature of AIH-2, only patients with AIH-1 were included in this study. In this study, some CHD patients had high mHAI scores. Whether HDV infection – as has been described for chronic HCV infection – can indeed trigger an AIH, is not fully elucidated.

On IFN-α therapy, HCV/LKM-1 positive patients experience increases in aminotransferase levels, occasionally of such magnitude

to warrant suspension of treatment (50, 51). Subgroup analysis of CHD patients showed no differences in NOSA titers irrespective of a previous IFN-α-therapy. In the current study, CHD patients with elevated NOSA titers did not experience relevant increases in aminotransferase levels upon IFN-α treatment. In our study, 43% of the CHD patients underwent treatment with pegylated IFN-α, although only 17% of IFN-α treated HDV patients showed a sustained response (HH-CHD8, HH-CHD26, HH-CHD42, Supplementary Table 3). These patients showed similar NOSA titers before and after treatment. Interestingly, in another recently published case of CHD with clinical and histological stigmata of AIH, therapy with the HBV/HDV entry inhibitor bulevirtide led to rapid normalization of immunoglobulin levels (52). Further studies are needed to assess whether such a therapeutic approach may lead to a substantial reduction of immunoglobulin levels. The literature does not indicate benefits in treating CHD patients displaying high NOSA titers with immunomodulating agents such as steroids (53, 54).

The median mHAI score was the highest in the AIH cohort, though not significantly compared to the CHD and CHB cohorts. Interestingly, the CHD patient with the highest mHAI score (10/18) also had highly elevated ANA titers and IgG levels. However, by histology, it is not possible to distinguish between viral hepatitis and AIH with certainty, and elevated NOSA as well as IgG levels were detected in many patients of the CHD cohort. Of note, NOSA patterns differed significantly in patients with AIH and viral hepatitis: the pattern of ANA autoantibodies in AIH patients was significantly more often homogenous than in the other two patient groups. Also, AIH patients displayed f-actin autoantibodies significantly more often than patients with viral hepatitis. If validated in larger studies, such differences in the NOSA titers and pattern may even be used to establish novel biomarkers to delimit the few patients with covert AIH from the majority of patients that demonstrate NOSA rather as an unspecific para-infectious immune phenomenon.

Notably, high NOSA titers might predict the development of autoimmune disease even after sustained HDV control (55). Arbuckle et al. found that ANAs with a dilution titer of 1:120 were present in 78% of lupus erythematosus patients studied 3–9 years before clinical manifestation (56). One patient of the CHD cohort also had cryoglobulins and was diagnosed with membranoproliferative glomerulonephritis (HH-CHD41, Supplementary Table 3). Additionally, prospective studies examining the presence of high autoantibody titers in an HDV cohort are needed to assess their significance as early predictors of autoimmune disease or as a singular para-infectious phenomenon. In this context, it seems important to investigate the prevalence of HDV infection in other autoimmune diseases. Further studies should assess the prevalence of additional clinical symptoms (arthralgias, sicca symptom) and autoantibodies typical of other autoimmune diseases. For example, anti-SSA and anti-SSB in Sjögren's syndrome, and cryoglobulins have been repeatedly found in patients with chronic hepatitis B, C, and E infection (34).

Possible mechanisms responsible for the immunopathology seen in chronic viral infections, in general, include molecular mimicry, impairment of regulatory T-cell activity, and polyclonal activation of B lymphocytes (31, 57, 58). Polyclonal activation of B cells seems to be a likely explanation of the correlation of IgG levels with HDV disease activity and severity. The persistence of elevated NOSA titers in non-viremic HDV patients could be due to the formation of persisting dysfunctional atypical memory B cells (61, 62). The role of B cells in CHD needs to be clarified in future studies.

In summary, autoantibodies are frequently detected as a para-infectious feature of CHD patients with unclear clinical significance.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Hamburger Ethikkammer. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JSZW, ML, and CW-N initiated and supervised the study. LH, SL, and JSZW wrote the first draft of the manuscript. SL, LH, and CW-N performed analyses and generated data. FH, MD, AWL, AZ, FP, and J-HB discussed the data and corrected the manuscript. All authors contributed to the manuscript and approved the final submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1169096/full#supplementary-material

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Apulian infectious diseases network: survey on the prevalence of delta infection among chronic HBV carriers in Apulia

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Background: The current prevalence and clinical burden of Hepatitis Delta Virus (HDV) infection in Apulia are unknown. This study aimed to define the current epidemiological scenario of delta infection and to detect difficulties in the diagnosis and clinical management of HDV patients in Apulia.

Methods: From May to September 2022, a fact-finding survey was conducted at eight Infectious Diseases Units of the Apulian region; each Unit was asked to complete a questionnaire on screening and diagnosis of HDV infection and demographic, virological, and clinical characteristics of HDV patients.

Results: A total of 1,461 HBsAg-positive subjects were followed up on an outpatient basis. Screening for HDV ranged from 30 to 90% of HBsAg + carriers in a single center. Overall, 952 HBsAg ± subjects (65%) were tested for HDV, and 80/952 (8.4%) were anti-HDV positive. Serum HDV RNA was detected only in 15/80 (19%) anti-HDV-positive subjects, and 12/15 patients (80%) were viremic. Sixty-five anti-HDV-positive subjects (81%) were from Italy; risk factors for HDV acquisition included the presence of HDV infection in the family (29/80 = 36%), drug addiction (12/80 = 15%), and co-infection with HCV or HIV (7/80 = 9%). Liver cirrhosis and hepatocellular carcinoma were diagnosed in 41 (51%) and 4 (5%) patients, respectively. Fifty-seven patients (71%) received nucleos(t)ide analog treatment.

Conclusions: The results of this survey show that HDV screening is variable and insufficient, thus real prevalence data on delta infection are lacking in Apulia. Moreover, the HDV RNA test is not available in most laboratories and is not provided by the national health system. These results underline the need for an organizational model to optimize the management of HDV patients throughout the Apulian region.

KEYWORDS

hepatitis B virus, Hepatitis Delta Virus, chronic viral hepatitis, hepatocellular carcinoma, epidemiology

Introduction

Hepatitis Delta virus (HDV) is responsible for a severe form of chronic viral hepatitis that can rapidly progress to liver cirrhosis and hepatocellular carcinoma (HCC) at higher rates than in monoinfected chronic hepatitis B (HBV) carriers (1–3).

In Italy, the progressive reduction of HBV infection in recent decades, mainly due to mandatory anti-hepatitis B vaccination, has also reduced HDV circulation (4–7). However, despite the declining prevalence, chronic hepatitis delta, due to its severity, remains a major public health issue for the National Health System.

Considering the severity of the disease and in view of new therapeutic perspectives (8–10), it is crucial to early identify and treat patients with hepatitis delta and to acquire the epidemiological data necessary for appropriate social welfare planning.

To date, prevalence data on delta infection are lacking in Apulia. Here we report the results of a survey conducted among chronic HBV carriers followed at eight Infectious Diseases Units of the Apulian region in order to define the current epidemiological scenario of delta infection, to detect difficulties in the diagnosis, and to lay the foundations for an organizational model for the diagnosis and care of the patient with hepatitis Delta in Apulia.

Methods

The Apulian Infectious Diseases Network includes eight Infectious Diseases Units distributed throughout the region. From May 2022 to September 2022, we carried out a Delta infection survey among all HBsAg carriers within the outpatient setting. Each unit was asked to complete a questionnaire containing the following information:

- The number of HBsAg-positive patients followed at each center
- The number of HBsAg-positive patients screened for HDV infection
- The number of HDV-positive patients
- The number of HDV-positive patients tested for serum levels of HDV RNA
- Risk factors for HDV infection
- Demographic characteristics of HDV-positive patients (age, sex, country of birth)
- Virological characteristics of HDV-positive patients (HBeAg/anti-HBe status, serum HBV DNA levels, serum HDV RNA levels, HDV genotype, coinfections with HCV and/or HIV)
- Clinical characteristics of HDV-positive patients (stage of liver fibrosis, presence of esophageal varices, diagnosis of HCC)
- Previous treatment with interferon
- Treatment with nucleos(t)ide analogs (NAs)

Each patient was given a progressive numerical code that included the province of the Center at which he or she was in follow-up. Virological and routine analyses were performed according to the best clinical practice of each clinical center. Anti-HDV, anti-HCV, and anti-HIV antibodies were tested by commercially available enzyme immunoassays. HDV-RNA was tested in two laboratories using an in-house PCR assay and more recently a commercially available assay (RoboGene HDV RNA quantification kit 2.0–lower limit of detection (LoD) 6 IU/ml. HDV genotype was assessed by genome sequencing in six patients with chronic delta hepatitis, enrolled in a clinical trial.

Statistical analysis

Sociodemographic, clinical, and virological features of the study population were collected and presented in terms of the number of subjects and percentages for categorical variables and of the mean (±Standard Deviation, SD) or median (Inter Quartile Range, IQR) for continuous variables in accordance with their parametric of non-parametric distribution. A p-value < 0.05 was considered statistically significant. Analysis was performed using the Jamovi package 2.3.2.

Results

A total of 1,461 HBsAg-positive subjects were followed up on an outpatient basis at the eight Infectious Diseases Units in Apulia. Screening for HDV was highly variable, varying from 30 to 90% of HBsAg+ carriers at a single center (Figure 1). Of the 1,461 HBsAg ± patients, only 952 subjects (65%) were tested for HDV and 80 of them (8.4%) were anti-HDV-positive.

Sixty-five anti-HDV-positive subjects (81%) were from Italy, while the remaining 15 (19%) were foreigners, mainly from Eastern Europe such as Romania, Albania, and Moldova (Figure 2).

Demographic, virological, and clinical characteristics of the 80 subjects with delta infection are shown in the Table 1. Fifty-four (68%) were men, and the median age was 58 years (range 28–85). Only 15 patients (19%) were tested for HDV RNA in two Apulian laboratories using an in-house PCR assay and more recently a commercially available quantitative assay; 12/15 patients were viremic: 4/7 were tested by a home-made PCR assay and 8/8 were tested using the commercial kit. In these latter patients, median serum HDV-RNA levels were 154,365.50 IU/ml (range 4,840–5,804,510 UI/ml). HDV genotype was available for six patients with chronic hepatitis D enrolled in a clinical trial, who were infected by genotype 1.

All 80 HBV/HDV patients were tested for HBV DNA, and detectable levels were found in 18/80 (23%) subjects. HBeAg positivity was found in only two patients of foreign nationality.

Among the risk factors for delta virus acquisition, the most frequent was the presence of HDV infection in the family (29 patients = 36%), other factors were drug addiction (12 patients = 15%), co-infection with HCV (five patients = 6%), or HIV (two patients = 3%). Liver cirrhosis was diagnosed in 41 patients (51%), and HCC development was reported in four patients (5%).

Thirty-three patients (41%) received one or more cycles of IFN without virological response and 57 (71%) were on treatment with NAs.

Data for both Italian and foreign subjects are shown in the Table 1. No significant differences were observed or described between the two groups.

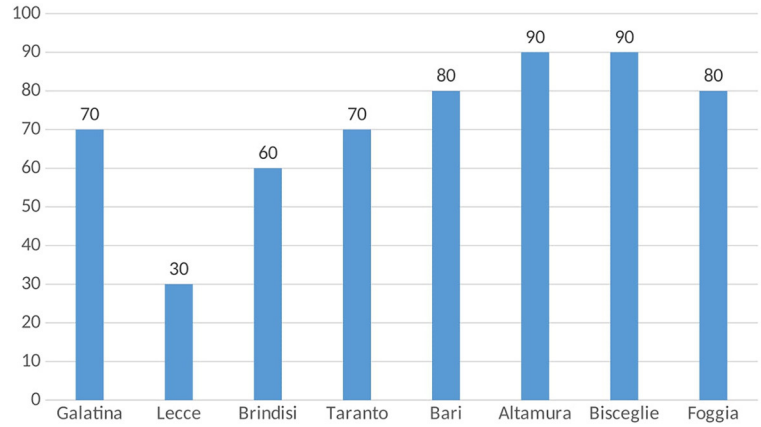

FIGURE 1
Percentages of HBsAg-positive patients screened for HDV infection across eight Apulian infectious disease units.

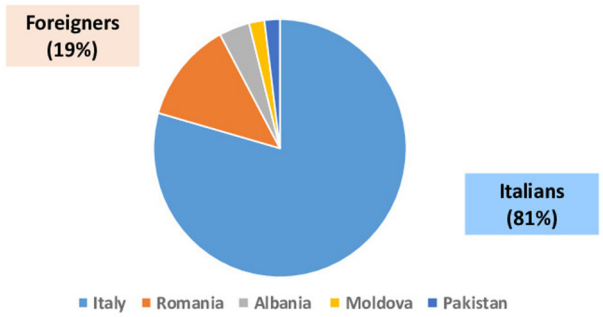

FIGURE 2
Country of origin of 80 HBsAg+/anti-HDV+ patients.

Discussion

In this survey conducted among HBsAg-positive subjects followed at eight Apulian Infectious Disease Units, the anti-HDV prevalence was 8.4%. This prevalence rate is similar to that recently reported in other Italian studies among HBsAg carriers with liver disease of varying severity followed in specialized centers (4–7). In a nationwide survey, Stroffolini and colleagues reported an anti-HDV overall prevalence of 9.9% (6.4% in Italian natives and 26.4% in non-natives) among 786 chronic HBsAg carriers consecutively referring to 9 tertiary centers in Italy (5). Moreover, data from the nationwide longitudinal PITER HBV/HDV ongoing cohort, reported an overall anti-HDV prevalence of 9.2% among 3,679 HBsAg carriers in care by 50 clinical centers (6, 7).

One of the main critical issues that emerged from the survey is that screening for HDV is highly variable and completely insufficient in some centers, due to the COVID-19 pandemic and possibly to the low awareness among healthcare providers of the persistent clinical and economic impact of delta hepatitis. The prevalence of HDV infection parallels the severity of liver disease and is higher in patients with liver cirrhosis or hepatocellular carcinoma (1). This survey does not provide the prevalence of delta infection among cirrhotic patients, however, the presence of cirrhosis in more than half of the patients with HBV/HDV infection confirms the severity of this double infection and the relevant pathogenic role of HDV.

Therefore, it is a priority to encourage the early identification of delta-infected individuals by implementing screening for HDV in all HBsAg-positive individuals with automatic laboratory detection of anti-HDV antibodies in all samples tested positive for HBsAg (reflex testing).

Another critical issue that emerged from the survey was the difficulty in accessing HDV RNA testing. The test is essential for documenting the presence of active delta infection and monitoring the effectiveness of therapy; however, it is not available in most laboratories and is not reimbursed by the national health system. According to national and international guidelines, HDV RNA should be quantified using well-standardized, validated real-time PCR assays (11).[1] Given that quantitative determination of HDV RNA is a fundamental requirement for an accurate diagnosis of ongoing HDV infection and to monitor antiviral treatment, identifying reference laboratories equipped to perform delta viremia is crucial (12).

Lastly, less than half of the patients performed one or more cycles of IFN therapy without benefit. In these patients, the new drugs will serve to broaden treatment options.

In conclusion, the results of this survey conducted among chronic HBV carriers followed at eight Infectious Diseases Units of the Apulian region, show that real HDV prevalence data in Apulia are lacking and that HDV screening is variable and insufficient. Moreover, the HDV RNA test is not available in most laboratories and is not provided by the national health system. These data underline the need for an organizational model to

1 https://www.webaisf.org/2023/03/14/indicazioni-operativeaisf-e-simit-per-la-diagnosi-e-la-gestione-clinica-del-paziente-affetto-da-epatitedelta/

TABLE 1 Demographic, virological, and clinical features of 80 HBsAg+/anti-HDV+ patients.

	Overall ($N = 80$)	Italians ($N = 65$)	Foreigners (15)	*p*
Sex, male (%)	50 (68)	46 (71)	8 (53)	*ns*
Age, years, median (range 28–85)	58	57	43	*ns*
Risk factors				
Intrafamiliar spread, *n (%)*	29 (36)	27 (42)	2 (13)	*ns*
Drug use, *n (%)*	12 (15)	12 (18)	0	
HIV infection, *n (%)*	2 (3)	2 (3)	0	
HCV infection, *n (%)*	5 (6)	5 (8)	0	
Tested for HDV RNA, *n (%)*	15 (19)	11 (17)	4 (27)	*ns*
HDV RNA positive, *n (%)*	12 (80)	9 (82)	3 (75)	
HBV DNA positive, *n (%)*	18 (23)	13 (20)	5 (33)	*ns*
HBeAg positive, *n (%)*	2 (3)	0	2 (13)	*ns*
Anti-HBe positive, *n (%)*	78 (97)	65 (100)	0	*ns*
Cirrhosis, *n (%)*	41 (51)	34 (52)	7 (47)	*ns*
HCC, *n (%)*	4 (5)	4 (6)	0	*ns*
Therapy				
- NAs, *n (%)*	57 (71)	46 (71)	11 (73)	*ns*
- IFN, *n (%)*	33 (41)	26 (40)	7 (47)	

N, number; HBV, Hepatitis B virus; HDV, Hepatitis D virus; HCV, Hepatitis C virus; HIV, Human Immunodeficiency Virus; NA, Nucleos(t)ide analog; IFN, Interferon; HCC, Hepatocellular carcinoma.

implement screening strategies, facilitate diagnosis, and optimize the management of HDV patients throughout the Apulia region.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Systemic cytokine and viral antigen-specific responses in hepatitis D virus RNA positive versus HDV RNA negative patients

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Background: Hepatitis B virus (HBV)/Hepatitis D Virus (HDV) co-infection increases the risk of severe liver disease compared to HBV mono-infection. Adaptive immune responses to HDV are weakly detectable, and the involvement of innate immunity in the progression of HDV-related liver fibrosis is suggested. We hypothesize that an overall innate immune activation in HBV/HDV co-infection plays a role in liver disease progression and also impacts virus specific T cell response.

Methods: Sixteen HBV/HDV-co-infected-patients (median age 42y/7F/6 Asian/4 White/6 Black/15 HBeAg-) and 8 HBV monoinfected-patients (median age 39y/4F/4 Asian/3 Black/1 White/HBeAg-) with median follow-up of 5 years were enrolled. Liver fibrosis was assessed by liver stiffness measurement (LSM, FibroScan®). Proliferation of CD3 + CD4+ T cells in response to viral antigens using CFSE assays and cytokine secreting monocytes was analyzed by flow cytometry.

Results: Of 16 HBV/HDV, 11 were HDV-RNA+ (HBV-DNA 0–1,040 IU/mL), 5/11 Interferon (IFN) + Nucleos/tide Analog (NA), 3/11 NA monotherapy, median ALT 77 U/L at the time of sample collection, median LSM of 9.8. In 5 HDV RNA–, median HBV DNA 65 IU/mL, 4/5 prior IFN and/or NA, ALT 31 U/L, and median LSM 8.5 kPa. In 8 HBV controls, median HBV-DNA, ALT, LSM was 69 IU/mL, 33 U/L,5 kPa, respectively. PBMC stimulation with HBV core antigen (HBcAg) and HDV antigen (HDAg) showed weaker CD3 + CD4 + T-cell proliferation in HDV-RNA+ vs. HDV RNA– and HBV-mono-infected patients ($p < 0.05$). In HDV-RNA+ patients, a correlation between ALT and TNF-α ($r = 0.76$, $p = 0.008$), higher IL-10 levels and increased proportion of CD14 + TNF-α+ cells were found.

Conclusion: In summary, during HBV/HDV coinfection, HDV RNA+ patients had weaker HBV and HDV specific responses, associated with increased TNF-α + monocytes irrespective of IFN treatment.

KEYWORDS

hepatitis D virus, cytokines, HBV/HDV co-infection, antigen-specific immune response, monocytes

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Introduction

The Hepatitis D virus (HDV) causes the most severe form of viral hepatitis in humans. It is estimated that among 296 million hepatitis B virus (HBV) surface antigen chronic hepatitis B (CHB), up to 15–20 million people are co-infected with HDV worldwide (1). HBV/HDV co-infection increases the risk of cirrhosis compared to HBV mono-infection with 70% of the co-infected cases progressing to end stage liver disease within 5–10 years (2). The underlying mechanisms of HDV induced liver disease pathogenesis are unclear. Similar to HBV, HDV is a non-cytopathic virus and the associated liver damage is thought to be an immune mediated injury (3). In chronic hepatitis D, HDV-specific adaptive response, particularly CD8+ T cell response is barely detectable and it had been recently discovered that HDV mutates to escape from the virus-specific CD8 T cell response (4, 5). One prior study found that HDV specific IFN-γ, IL-2 CD4+ T cell response is present in interferon alpha (IFN-α) induced and spontaneous viral clearance cases (5). Studies in mouse models of HDV infection and in 31 patients, have shown that innate immune responses mediated by monocytes and natural killer (NK) cells are implicated in accelerating liver damage in HBV/HDV co-infection (6–8). Further, in an immunodeficient humanized mouse model of HBV/HDV infection it was noted that co-infected mice showed increased inflammatory response which was correlated with increased liver damage compared to uninfected and HBV mono-infected mice (6). Recent studies using HBV/HDV cell culture models show that HDV but not HBV induces an innate immune response, yet the IFN induced response did not have a significant effect on HDV replication (9). Overall, these studies point out a central role of innate immune responses in HBV/HDV co-infection. We hypothesized that an overall innate immune activation in HBV/HDV co-infection drives liver damage and provide data on HDV-specific and additionally HBV specific T cell responses in co-infection.

Patients and methods

In total 16 HBV/HDV co-infected carriers were prospectively recruited ($n = 11/16$ HDV-RNA positive) from the University of Calgary (U of C) Liver Unit. In addition, 8 HBV mono-infected cases (with similar HBV DNA levels, <2–3 log IU/mL) were enrolled. Clinical characteristics of the patients in this study are outlined in Table 1. All subjects provided informed written consent to participate according to the guidelines of the 1975 Declaration of Helsinki. This study was approved by the U of C conjoint health research ethics board, CHREB (Ethics ID 16636). Clinical data and laboratory assays such as serum HBV DNA (according to clinical PCR, Abbott Architect lower limit of detection 10 IU/mL or ~ 50 virus copies per mL) was collected. Additional HBV serology (i.e., HBsAg, HBeAg, anti-HBeAg) was determined clinically with commercial chemiluminescent microparticle immunoassays (Abbott Architect; quantitative anti-HBc II and anti-HBs). HBV genotyping was done by in-house nested PCR as previously published (10). HDV-RNA testing was performed at National Microbiology Laboratory (NML), Winnipeg. Quantitative HDV RNA was measured by real-time RT-PCR method (linear range of 3.1 log10 to 10.4 log10 copies/mL (11).

TABLE 1 Clinical characteristics of HBV/HDV and HBV patients enrolled in the study.

	HBV ($n = 8$)	HBV/HDV Gr.1 (HDV RNA+) ($n = 11$)	HBV/HDV Gr. II (HDV RNA−) ($n = 5$)
Median age in years (median IQR)	39 (18.5)	39 (14)	45 (29)
Sex	4 M/4F	5F/6M	1F/4M
Ethnicity	4 Asian 3 Black 1 White	3 Asian 4 Black 4 White	4 Asian 1 White
Median HBV-DNA (IU/mL)* [Range, median IQR]	69.5 [13–226, 164.2]	50 [0–104, 43]	65 [13–103, 58]
Median HDV-RNA (copies/mL) [Range, median IQR]	N/A	1.4×10^6 [3.3×104-3.1×10^8, 1.3×10^7]	N/A
Median ALT (U/L) [Range, median IQR]	33 [8–61, 49.75]	77 [23–469, 60]	31 [12–88, 40]
Median LSM (kPa) [Range, median IQR]	5 [3.3–6.1, 3.1]	9.8 [5.3–42, 7.4]	8.5 [5.5–21.3, 9.6]
Treatment Status	Untreated	5 IFN + NA 3 NA monotherapy 3 untreated	3 IFN + NA 1 NA monotherapy 1 untreated

Gr. I: HDV-RNA+ patients, Gr. II: HDV-RNA−patients. *1 IU/mL = ~5.2 virus genome copies/mL.

Isolation of peripheral blood mononuclear cells

PBMC were separated by density gradient centrifugation from ~40 mL of heparin anti-coagulated whole blood, and ~ 10^7 cells/vial were cryopreserved. Serum isolated from 10 mL of whole blood was stored at-80oC.

Analysis of serum cytokines

Cytokines were assessed using Human Focused 13-Plex Discovery Bead Based Immunoassay (Luminex technology) by Eve technologies, Calgary, Canada. Samples were run in duplicates. Serum and culture supernatants were used as sample types for the assay.

Antigen specific proliferation assays

Bulk PBMC were used to perform the described assays. Fresh or cryopreserved PBMC (in 5 patients) were labeled with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, BD Horizon,

San Diego, CA) in DPBS for 10 min at 37°C. Cells were then centrifuged at 300 g for 10 min, washed with Rosewell Park Memorial Institute (RPMI), reaction was stopped with complete RPMI (with 10% fetal bovine serum, FBS) and then suspended in complete RPMI. Labeled PBMC were stimulated with 5 μg HBsAg (adw), 5 μg HBcAg, and HDAg (American Research Products, Waltham, MA) in (RPMI) 1,640 supplemented with 10% FCS, 2 mmol/L glutamine and Pen-Strep antibiotic solution (Sigma, Oakville, ON). Phytohemagglutinin-M (PHA-M, Carlsbad, CA) 5 μg/mL or anti-CD3 (1 μg/mL)/anti-CD28 (5 μg/mL) (BD Biosciences, San Jose, CA) stimulated cells served as positive control. Unstimulated DMSO treated cells were negative controls since CFSE was dissolved in DMSO. Cells were cultured in triplicates and plates were incubated at 37°C with 5% CO_2 for ~7 days. Cell proliferation was assessed on day 7 or 8. Stimulation index (SI) was calculated as % CFSE low cells in stimulated cells / % CFSE low cells in the unstimulated (DMSO) control as per our previously established protocols (12). SI values >2 were considered as positive for antigen specific proliferation. Supernatants were collected at 72 h, stored in-80°C and analyzed in a Luminex assay to study cytokine levels.

Cytokine release by monocytes

Approximately 10^6 fresh PBMC were stimulated with 5 μg/mL lipopolysaccharide (LPS, Sigma, Oakville, ON) for 18 h. Unstimulated DMSO treated cells were used as negative controls. After 2 h, 1 μg/μL per million cells Golgi-Plug was added. After 16 h, cells were washed and stained with FVS510 to exclude dead cells and with anti CD14-BV605 to identify monocytes. Cells were washed again, fixed, and permeabilized with the Cytofix/Cytoperm Kit, stained with PE conjugated antibodies against TNF, IL-1β, IL-10 for 30 min at 4° C, washed, re-suspended in PBS, and immediately analyzed by flow cytometry. A positive response was defined as >2 fold the DMSO background response. All antibodies and reagents used for flow cytometry were purchased from BD Biosciences, Mississauga, ON unless mentioned otherwise.

Statistical analyses

Data were analyzed using Graphpad Prism 7 (La Jolla, CA). Demographic, clinical and laboratory parameters were compared using measures of central tendency, Mann–Whitney U test and Kruskal-Wallis test with post-hoc Dunn's test for multiple comparisons was used to compare flow data. Non-parametric Spearman's rank correlation test was used for correlation analysis; p values <0.05 were considered significant.

Results

Summary of clinical data

In total, 16 HBV/HDV co-infected cases were enrolled in the study. Of these $n = 11$ were HDV-RNA positive at the time of sample collection with 5 on IFN/NA combination, 3 on NA monotherapy and 3 untreated (HBV/HDV Group I, HDV-RNA+ median HDV-RNA 1.4×10^6 copies/mL). In comparison, 5/16 had undetectable HDV-RNA (HBV/HDV Group II, HDV-RNA−) with 3 previously treated with IFN + NA therapy, 1 on NA monotherapy and 1 untreated. It is known that co-existence of HBV and HDV, usually results in HBV suppression and HDV dominance (13). In our cohort of HBV/HDV co-infected individuals, median HBV levels were ~ 60 copies/mL, thus as a control, 8 HBV mono-infected patients (HBV) with similar HBV-DNA levels (69 copies/mL) were enrolled (see Table 1, Supplementary Table 1).

Estimation of serum cytokines and correlation to alanine transaminase levels

We tested 13 pro-inflammatory, Th1 and Th2 cytokines in co-infected and mono-infected patients. Of note, IL-10 was significantly increased in the HDV-RNA+ group compared to the other groups (Figure 1A). We observed an increase in IL-1β in HBV/HDV co-infection vs. HBV mono-infection ($p < 0.05$) (Figure 1B). A similar trend was also noted for TNF-α (Figure 1C). Interestingly, all of the 13 cytokines studied were elevated although non-significant increase in the HDV-RNA+ patients compared to HDV-RNA− and HBV mono-infected groups (Table 2). Several studies have studied cytokines as biomarkers of liver disease. We therefore performed correlation analysis between ALT and each of the cytokines. Of all the soluble markers investigated, TNF-α, IL-1β, and IL-10 were significantly and positively correlated with ALT levels (Figures 1D–F).

Cytokine production by monocytes

A previous study highlighted the role of Inducible Protein (IP) -10 producing monocytes from peripheral blood and CD206+ macrophages in the liver in HBV/HDV co-infection and liver fibrosis and/or inflammation (5, 7). Further, monocyte activation and cytokine release is implicated in viral hepatitis and viral co-infections (14, 15). Thus, we explored activation induced (LPS) cytokine secretion by CD14+ monocytes. Although, no difference was noted for IL-10 and IL-1β, a significant increase in the proportion of CD14 + TNF+ PBMCs was noted in the HDV-RNA+ group vs. – mono-infected group and a similar trend was noted for HDV-RNA+ vs. HDV-RNA−group (Figures 2A–D).

HBV and HDV specific CD4 T-cell response in HBV/HDV co-infection using bulk PBMC

We hypothesized that presence of activated monocytes and systemic inflammation contributes to enhanced T-cell responses (16). We examined HBsAg, HBcAg, and HDAg specific responses *ex vivo* using PBMC from co-infected and mono-infected individuals. Surprisingly, we found reduced HBcAg and HDAg specific proliferation of CD4+ T cells in HDV-RNA+ patients compared to HDV-RNA−group ($p < 0.05$) (Figures 3A–D).

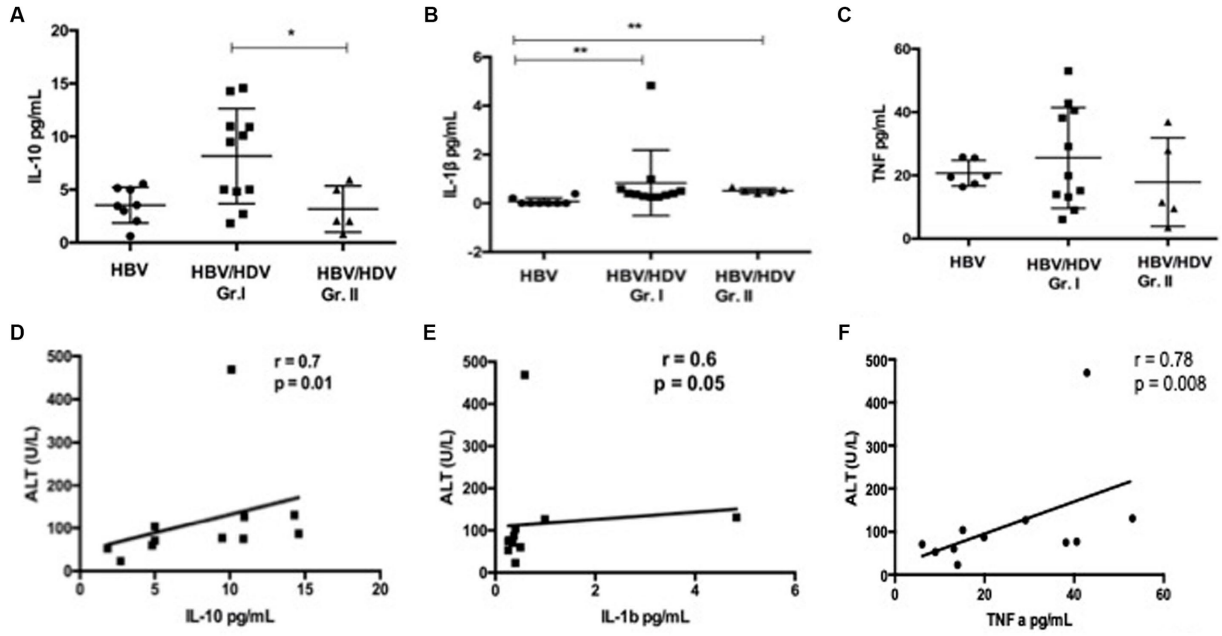

FIGURE 1
Comparison of serum cytokines in HDV patients **(A)** IL-10, **(B)** IL-1β, and **(C)** TNF-α in pg./mL in HBV/HDV Gr. I (HDV RNA+) vs. HBV/HDV Gr. II (HDV RNA–) patients. Correlation analysis between ALT and serum cytokines, **(D)** IL-10, **(E)** *IL*-1β, and **(F)**. TNF-α in HDV RNA+ patients. * $p < 0.05$, Kruskal–Wallis test.

TABLE 2 Serum cytokines and chemokines in HBV/HDV co-infection and HBV mono-infection.

	HBV	HBV/HDV Gr. I (HDV RNA+)	HBV/HDV Gr. II (HDV RNA–)
IFN-γ	1.3 ± 0.2	5 ± 3.8	1.4 ± 0.2
IL-2	0.58 ± 0.12	1.9 ± 0.9	0.6 ± 0.04
IL-4	1.9 ± 0.49	3.8 ± 1.9	1.07 ± 0.3
IL-5	0.15 ± 0.06	0.8 ± 0.27	0.4 ± 0.03
IL-6	0.6 ± 0.16	0.8 ± 0.4	0.47 ± 0.19
IL-8	4 ± 0.9	32 ± 13.63	9.5 ± 1.3
IL-12	0.4 ± 0.18	3.4 ± 2.4	0.4 ± 0.2
IL-13	0.63 ± 0.16	0.89 ± 0.44	0.47 ± 0.19
GM-CSF	3 ± 0.33	13.65 ± 8.7	3.5 ± 0.3
MCP	2.1 ± 0.89	16.6 ± 10	0.8 ± 0.2

Th1 and Th2 cytokines in PBMC culture supernatants

Levels of IL-2, IL-4, IL-5, TNF-α, and IFN-γ in the PBMC culture supernatants from HDV-RNA+ cases were lower than in HDV-RNA− and HBV mono-infected cases, especially in response to HBcAg and HDAg. In 8/11 HDV-RNA+ patients, Th1 cytokines – IL-2, IFN-γ were undetectable in response to HBcAg (Table 3).

Summary of antigen specific response in the 16 HBV/HDV co-infected patients

Based on cytokine release in antigen stimulated culture supernatants and proliferation experiments all the HDV-RNA negative cases ($n = 5$) showed HDV and HBV (especially HBcAg) specific T cell responses analyzed in bulk PBMC. 6/11 HDV-RNA positive cases did not show responses to either HBV or HDV antigens. These results show differences in viral antigen specific immune response in HDV-RNA positive vs. negative patients. Table 4 reports T cell responses for $n = 16$ HBV/HDV co-infected patients.

Discussion

Chronic hepatitis D is the most severe form of viral hepatitis with very limited treatment options and poorly understood immunological aspects of disease progression (3). In this study, we show that an increased inflammatory response was observed in "active" HDV-RNA+ patients compared to HDV-RNA−cases in association with weak HBV and HDV specific T-cell response, irrespective of IFN treatment history.

The current data suggests that in cases with HDV viremia, an overall immune activation exists. Further, TNF-α, IL-1β, and IL-10 levels correlated with ALT suggesting a role in liver damage in the setting of co-infection. Townsend et al. also reported increased systemic cytokines in the HDV patients with a high viral load (6.1–6.4 HDV-RNA, IU/mL) vs. mild HDV cases (1.5–6 IU/mL) (17). A previous study in an immuno-deficient mouse model of HBV/HDV

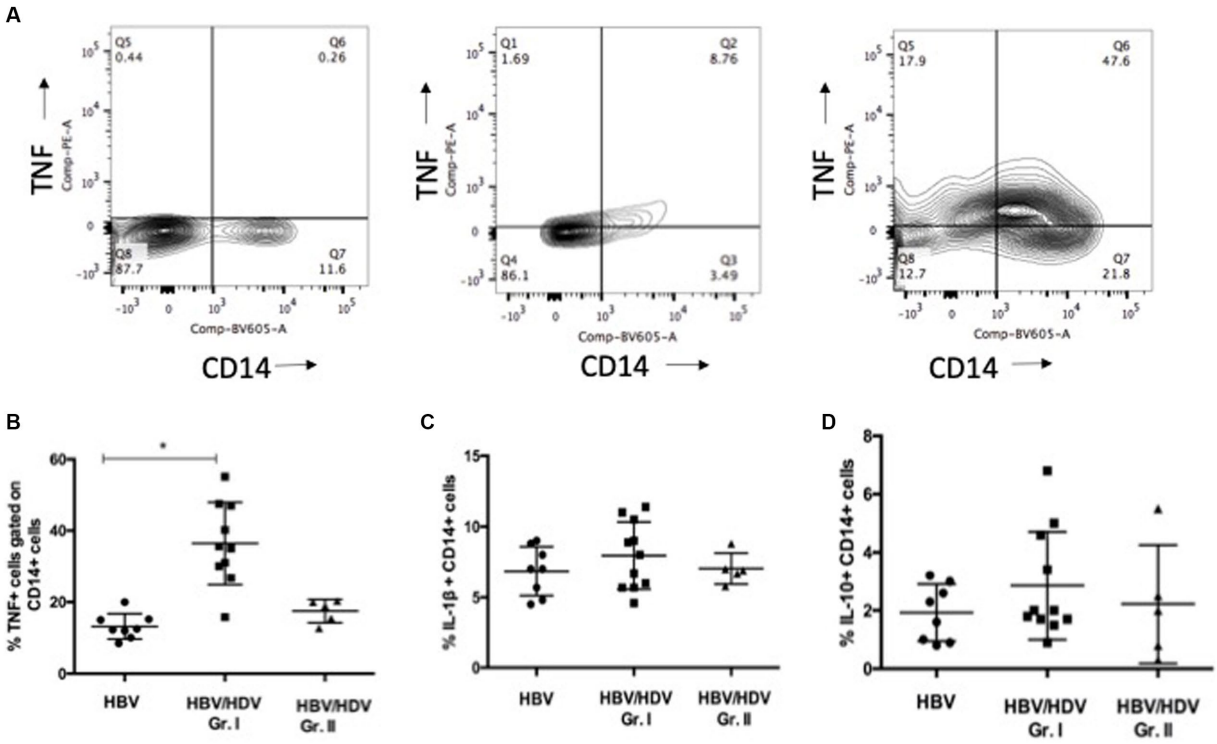

FIGURE 2
Comparison of CD14+ TNF+, CD14 + IL-1β, CD14 + IL-10+ monocytes in HBV/HDV Gr. I vs. other groups. **(A)**. Representative plots of CD14 + TNF+ cells in negative control (DMSO control), HBV/HDV Gr. II (HDV RNA+) and HBV/HDV Gr. I (HDV RNA –) patients. **(B)**. Frequency of CD14 + TNF+ cells. **(C)**. Frequency of CD14 + IL-1β cells. **(D)**. Frequency of CD14 + IL-10+ cells in the three categories. Individual values have been plotted. * $p < 0.05$, Kruskal–Wallis test.

co-infection showed high levels of basal pro-inflammatory and pro-fibrogenic cytokine levels which may directly contribute to liver disease progression (6). Interestingly, in an adenovirus HBV/HDV mouse model, the use of a TNF-α agonist resulted in significant reduction in HDV related liver damage (18). We noted an increased proportion of TNF producing monocytes in response to LPS treatment in HDV-RNA+ patients. Another study reported IP-10 release from monocytes when stimulated with HDV peptides (5). In human liver tissue ($n = 15$), intrahepatic CD14+ cells were found to be associated with pathological inflammation (HBV, HBV/HDV, HCV). Intrahepatic leucocytes from these livers produced high levels of TNF-α, IL-1β and IL-6 compared to healthy livers (7). Ito et al. elegantly proved in HBsAg transgenic/TNF double knockout mice that TNF-α produced by intrahepatic non antigen specific inflammatory cells is critical in the development of lethal liver disease (19). Taken together, these findings highlight the role of pro-inflammatory monocytes in liver injury and also suggest that TNF+ monocytes may be a potential biomarker in predicting risk of liver disease in HBV/HDV co-infection. In chronic hepatitis C, a decrease in LPS stimulated TNF production by monocytes was linked to poor disease outcome (20). In contrast, an increase in PBMC CD11b + macrophage frequency and phagocytic activity has been reported in fulminant hepatitis E (21). Decreased T-cell response in association with LPS activated monocytes has been previously reported. The state of inflammation in HDV-RNA+ patients is similar to the systemic inflammatory milieu in obesity (i.e., enhanced TNF levels and also conversion of memory T cells to naïve T cell subsets) (22, 23). These reports and our data point out toward differential hepatitis D virus specific monocyte functions.

HBV/HDV is a dynamic disease with fluctuating patterns of HBV and HDV dominance over time. Thus, it is crucial to study HBV specific response as well as HDV specific immune response. We found weak HBsAg and HBcAg specific CD4+ T-cell proliferation in HDV-RNA+ cases vs. HDV-RNA−and HBV mono-infected patients. Similarly, reduced HDV specific response was noted in HDV-RNA positive vs. negative cases. Nisini et al. found that PBMC from 8 of 30 patients (27%) significantly proliferated in response to HDAg. Another study showed that 12/46 (28%) Studies have shown that ~28% (8/30 and 12/46)of HBV/HDV patients show proliferation in response to HDAg (24, 25). Prior immunologic studies in CHB showed an increase of HBcAg/HBeAg-specific T cell proliferation before and during ALT flares along with an increased production of Th1 cytokines IFN-γ and IL-2 was noted (26, 27). In our study, we found that Th1 response was barely detectable in HDV-RNA+ cases despite ALT flares. These data suggest a differential regulation of T-cell response in HBV/HDV co-infection than mono-infection. Severe liver damage in HBV/HDV co-infection in chimpanzees correlated with a lack of Th1 response (28). Similar to Grabowski et al., we noted that HDV-specific IFN-γ and IL-2 responses were detectable in all HDV-RNA−patients and 4 HDV-RNA+ patients after *in vitro* stimulation of PBMC with HDAg (5). It would be interesting to note the dynamics of T-cell response in serial samples collected from HDV-RNA positive patients to gain further insights on viral clearance and viral relapse in HDV-RNA negative cases. Sorting and

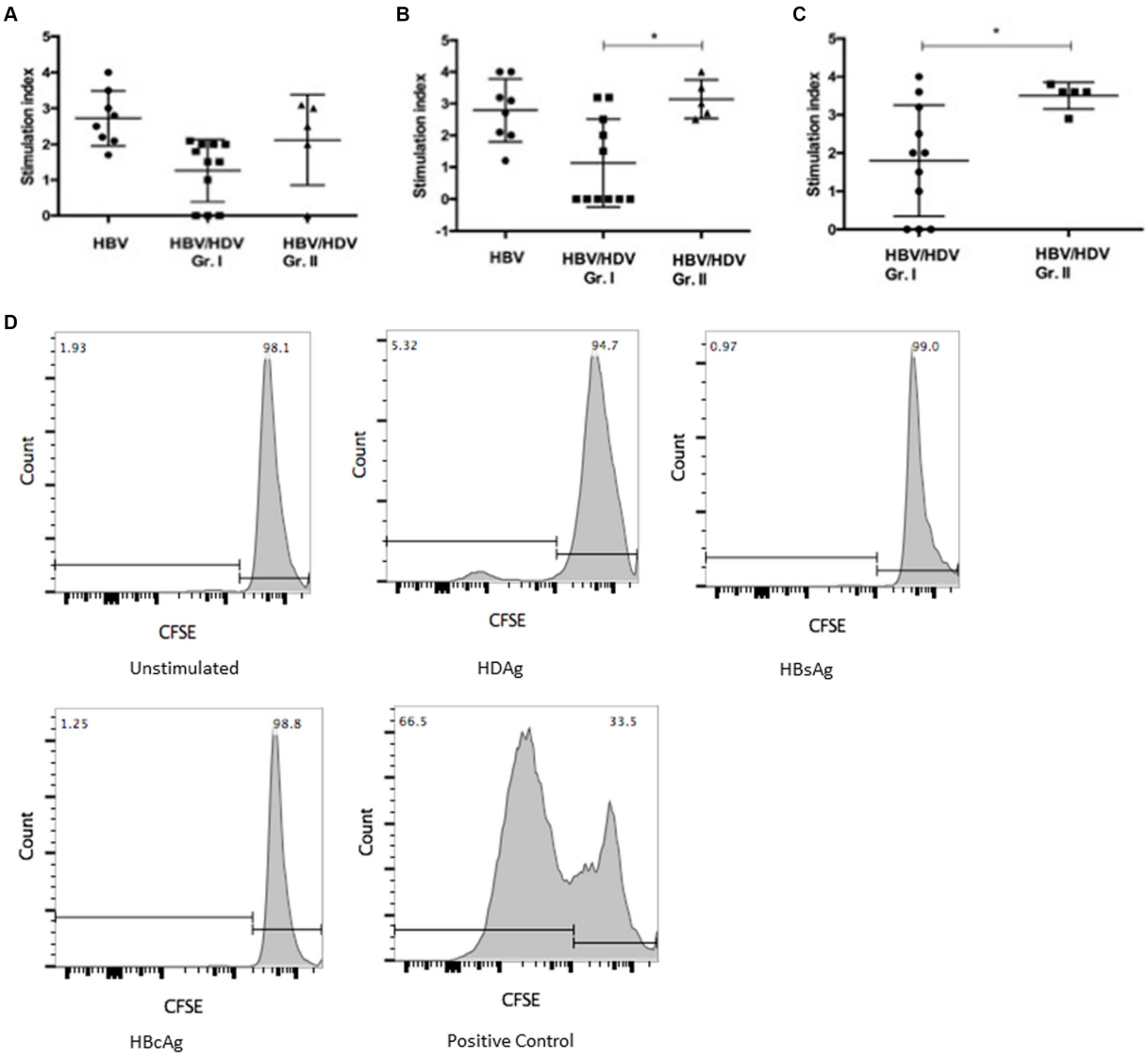

FIGURE 3
Comparison of T-cell proliferation in PBMC from HBV/HDV co-infected patients and HBV mono-infected patients in response to viral antigen stimulation **(A)** HBsAg, **(B)** HBcAg, and **(C)** HDAg specific CD3 + CD4+ T-cell response in HBV/HDV Gr. I (HDV RNA+) vs. HBV/HDV Gr. II (HDV RNA–) patients. * $p < 0.05$, Kruskal–Wallis test and Mann–Whitney test. Values represent mean ± standard deviation. **(D)** Representative CFSE proliferation prolife in a HDV-RNA+ patient (HBV/HDV Gr. I) in response to unstimulated, HDAg, HBsAg, HBcAg, and mitogen stimulated positive control.

co-culture of monocytes and T cells to clearly delineate the role of monocytes in modulating T-cell mediated response in HBV/HDV co-infection.

The current single center study is limited by small sample size of 16 HBV/HDV co-infected patients ($n = 11$ HDV RNA+ and 5 HDV RNA−), and inability to assess intrahepatic immune response with liver biopsy. Hepatitis Delta is an orphan disease and there is limited clinical study on immunological aspects of HBV and HDV mediated liver fibrosis progression. A German study of 41 HDV/HBV patients showing chronic HDV infection engages the mucosal-associated invariant (MAIT) T cell compartment causing activation, functional impairment, and subsequent progressive loss as the potential cause of HDV-associated liver disease progresses, although the study could not link this data to long-term clinical outcomes (29). The authors hypothesized that cytokine driven (IL-12 and IL-18) activation could lead to cell death and peripheral loss of MAIT cells, like other inflammatory conditions including autoimmune hepatitis. Landahl et al., analyzed HDV specific T cell responses at single peptide level in 32 HDV infected patients in Italy (vs. 6 monoinfected) following *in-vitro* stimulation with 21 overlapping peptides, and found >1 T-cell response in 50% tested but no difference in HDV RNA positive vs. negative patients (30). A study by Kefalakes et al., found that in 28 HBV/HDV co-infected patients activated but not terminally differentiated HDV-specific CD8 T-cell response correlated with liver disease progression. Furthermore, in half of the patients, HDV clearance by CD8 T cells did not occur and reduced T cell activation in association with escape variants of HDV was noted (31). Loss of cytokine production and proliferation has been attributed to T cell

TABLE 3 Th1 and Th2 cytokines in HBV/HDV co-infection and mono-infection in response to HBV and HDV proteins *in-vitro*.

HBsAg	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-2 (pg/mL)	IFN- γ (pg/mL)	TNF-α (pg/mL)
HBV/HDV Gr. I	4.5 (3.4)	6 (0.8)	2.8 (1)	8.1 (2)	10.2 (0.9)
HBV/HDV Gr. II	5.4 (0.6)	7.2 (1)	2.5 (1.4)	5.6 (0.2)	11.5 (0.5)
HBV	4.6 (1)	8.4 (1.1)	3 (0.6)	9.6 (1.1)	10.9 (2.5)

HBcAg	IL-4	IL-5	IL-2	IFN- γ	TNF-α
HBV/HDV Gr. I	2.5 (0.2)	5.5 (0.4)	2.4 (2.3)	3.5 (4.4)	15 (3.9)
HBV/HDV Gr. II	4.7 (0.6)	8 (1.9)*	5.1 (2)*	7.8 (2.2)*	12.5 (2.5)
HBV	3.5 (0.5)	4.2 (1)	3.5 (0.7)	2.1 (0.9)	11.4 (3.5)

HDAg	IL-4	IL-5	IL-2	IFN- γ	TNF-α
HBV/HDV Gr. I	3.5 (1.2)	2 (0.8)	2.8 (1)	1.9 (0.6)	9 (1.2)
HBV/HDV Gr. II	6.7 (1)*	5.7 (1.1)*	6.2 (0.4)*	5 (1)**	13 (2.2)

*$p < 0.05$, ** <0.01 by Kruskal–Wallis test.
Values in parentheses represent standard deviation. Values represent pg/mL of cytokines stimulated – unstimulated culture supernatants.

TABLE 4 Virus specific T-cell response in patients with HBV/HDV co-infection.

Sample ID #	Date of sample collection	HDV RNA status at the time of sample collection	Response to HDAg	Response to HBsAg	Response to HBcAg
103	January 2017	Negative	Yes	Yes	Yes
104	January 2017	Negative	Yes	Yes	Yes
177	May 2017	Positive	No	No	No
218	June 2018	Negative	Yes	No	Yes
323	July 2016	Positive	No	Yes	No
325	May 2017	Positive	No	No	No
332	May 2017	Positive	Yes	Yes	Yes
336	January 2017	Positive	No	No	No
342	August 2018	Negative	Yes	Yes	Yes
347	May 2017	Positive	Yes	Yes	Yes
353	July 2017	Positive	Yes	No	Yes
358	August 2017	Positive	Yes	No	Yes
360	October 2017	Positive	No	No	No
364	October 2017	Positive	No	No	No
370	December 2017	Positive	No	No	No
395	August 2018	Negative	Yes	Yes	Yes

exhaustion in HCV and HBV infections but is widely understudied in HBV/HDV co-infections and may be an area for future investigation in our HBV/HDV patient cohort (32).

Conclusion

There is limited data on immunological aspects of HBV and HDV mediated liver fibrosis progression. The current study provides further detail characterization of the functionality of HBV and HDV specific T cell responses and cytokine responses compared to HBV monoinfection, linked to long-term clinical and virological outcomes. Hepatitis Delta co-infected patients with viremia show higher activation induced TNF alpha release by monocytes in association with weak HBV and HDV specific T-cell responses compared to HDV-RNA negative patients irrespective of anti-viral treatment status. The study contributes to limited data on immunopathogenesis of hepatitis Delta virus Infection.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the University of Calgary Conjoint Health Research Ethics Board, CHREB (Ethics ID 16636). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SSJ: study design, execution, data analysis, and preparation of manuscript. CSC: study design, manuscript draft, and patient recruitment. MS: patient recruitment. NHP: data analysis, manuscript review, and feedback. CO and KF: HDV genotyping data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1125139/full#supplementary-material

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