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Preliminary Evidence for Hepatitis Delta Virus Exposure in Patients Who Are Apparently Not Infected With Hepatitis B Virus

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Hepatitis delta virus (HDV) is a defective human virus that lacks the ability to produce its own envelope proteins and is thus dependent on the presence of a helper virus, which causes its surface proteins to produce infectious particles. Hepatitis B virus (HBV) was so far thought to be the only helper virus described to be associated to HDV. However, recent studies showed that divergent HDV-like viruses can be detected in fishes, birds, amphibians, and invertebrates without evidence of any HBV-like agent supporting infection (reviewed in Maya and Ploss[(1)]). Another recent study demonstrated that HDV can be transmitted and propagated in experimental infections *ex vivo* and *in vivo* by different enveloped viruses unrelated to HBV, including hepatitis C virus (HCV), flaviviruses like dengue and West Nile virus, and vesiculovirus.[(2)] Altogether, these results suggested that hepatitis D infection may, in theory, occur in patients carrying either virus.[(2)] These observations prompted us to search for HDV infection among patients who are HCV infected and in geographical regions with high HDV endemicity.

The exact prevalence of HDV infection in Venezuela is unknown, but outbreaks of fulminant HDV infections have been reported in indigenous populations from the Amazon basin and Western Venezuela.[(3)] The high prevalence of HDV infection among these indigenous populations might have favored dissemination of HDV infection among other inhabitants in the country. Here, we investigated the possible HDV exposure in a cohort of Venezuelan patients infected with HCV.

Clinical Observation

The sera of patients who were HCV infected were analyzed in 2004-2005 and 2014-2015 when requesting determination of HCV genotype for treatment.[(4)] All patients (n = 160) were HCV RNA-positive (Fig. 1) and were treated with a combination of pegylated interferon plus ribavirin. No information is available on the success of the treatment. The sera from 2 patients were positive for anti-HDV antibodies, as tested by enzyme-linked immunosorbent assay and further confirmed using a LIAISON-XL immunoassay, but were negative for all serological or molecular markers of

Abbreviations: AU, arbitrary unit; ddPCR, droplet digital PCR; HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis delta virus; nts, nucleotides; OD, optical density; qPCR, real-time quantitative PCR.

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HBV—hepatitis B surface antigen (HBsAg), anti-hepatitis B core (HBc) antibodies, and HBV DNA—as assessed by real-time quantitative PCR (qPCR) and droplet digital PCR (ddPCR). HDV RNAs were detected in one sample by nested PCR.[3] The purified PCR fragments were sequenced and revealed HDV RNA of genotype 1, which is frequently found in Venezuela together with HDV genotype 3 (Fig. 2A).[3] Compared with other genotype 1 HDV sequences, the C-terminal region of the HDV L-antigen, harboring essential assembly and envelopment functions, was conserved in the Venezuelan isolate (Fig. 2B).

Discussion

Our observation provides preliminary evidence of HDV exposure in patients who were chronically

160 HCV RNA positive sera from Venezuela
aged 12-85 years (mean 48.5 ± 16.8 years)
HCV genotypes: 50% G1, 43% G2, 6.4% G3, 0.7% G6

2/160 anti-HDV positive (1.25%)

1/2 HDV RNA positive by nested RT-PCR

ID	Age	Sex	HCV genotype	HBsAg[1]	Anti-HBc[1]	HBV DNA[2] (qPCR and ddPCR)	Anti-HDV Diasorin OD/Cutoff[3]	Anti-HDV LIAISON®[4]	HDV RNA[5]	HDV genotype	Risk factor
3712	52	F	2	-	-	-	0.404	1.96 AU/ml	+	1	unknown
3724	61	F	1a	-	-	-	0.156	7.38 AU/ml	-	-	unknown

FIG. 1. Detection of HDV exposure among 160 Venezuelan patients who were HCV infected. [1]HBsAg (SURASE B-96; General Biologicals Corporation and Architect, Abbott) and anti-HBc (ANTICORASE B-96; General Biologicals Corporation and Architect, Abbott) serological markers were tested by commercial immunoassays, and two sera were found negative by all the tests. [2]No HBV DNA (lower limit of detection [LLOD] 50 copies/mL) was detected by qPCR (IQ SYBR Green, Bio-Rad, using primers located in the HBV S gene (sense [Ss: 5′-GTG TCT GCG GCG TTT TAT CA-3′, nts 379-398 bp] and antisense [Sas: 5′-GAC AAA CGG GCA ACA TAC CTT-3′, nts 456-476 bp]) nor by ddPCR (LLOD 10 copies/mL) in either sample. ddPCR was performed on a QX100 Droplet Digital PCR (Bio-Rad) using the Taqman Pathogen Detection assay ID Pa03453406_s1 from Thermo Fisher Scientific (HBV detection, primer mix for amplification of P, S/P, and X genes). [3]OD/cutoff average ratio of anti-HDV antibody assays obtained by enzyme-linked immunosorbent assay (ETI-AB-DELTAK-2, DiaSorin). Negative control OD ≥ 0.6; positive control OD ≤ 0.008; cutoff OD = 0.4. OD/cutoff average ratio of negative samples = 2.339. [4]AU obtained by anti-HDV antibody testing with LIAISON murex XL Anti-HDV, DiaSorin (Ref 311260, indirect enzyme immunoassay). Negative control < 0.02 AU/mL; positive control > 13 AU/mL; cutoff = 1.0 AU/mL. [5]HDV RNA was detected by nested PCR using the following primers: 5413: 5′-GCC CAG GTC GGA CCG CGA GGA GGT-3′ (nts 858-881), 8276: 5′-ACA AGG AGA GGC AGG ATC ACC GAC-3′ (nts 1312-1289), 5414: 5′-GAG ATG CCA TGC CGA CCC GAA GAG-3′ (nts 883-906), and 5415: 5′-GAA GGA AGG CCC TCG AGA ACA AGA-3′ (nts 1288-1265). Abbreviations: AU, arbitrary unit; G, HCV genotype; OD, optical density; nts, nucleotides.

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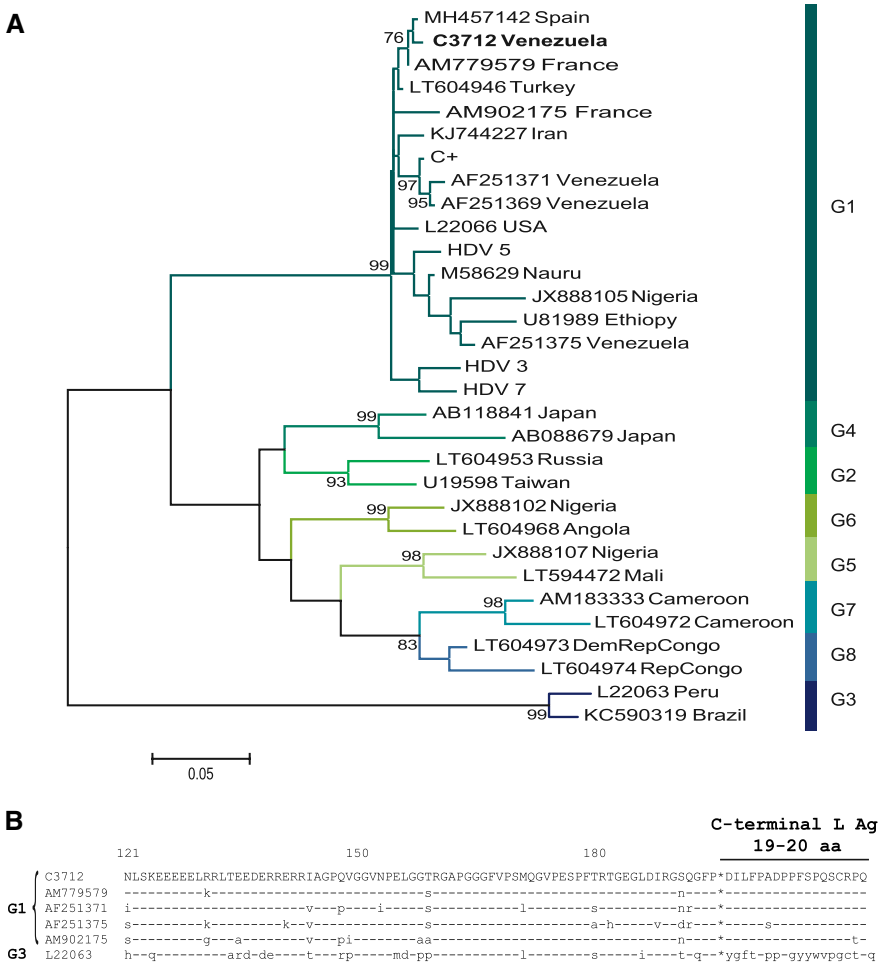

FIG. 2. (A) Phylogenetic analysis of Venezuelan HDV isolate by Maximum Likelihood method (360 nt). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (1,000 bootstrap replicas). A discrete gamma distribution was used to model evolutionary rate differences among sites (3 categories +G, parameter = 1.3600). Sequences are shown by their accession number and country of origin. Genotypes are shown in different colors. The Venezuelan HDV isolate retrieved in this study (C3712, accession number MT274327) is shown in bold. Sequences from HDV isolates from patients whose RNA was simultaneously extracted and DNA amplified along with the Venezuelan isolate are included and referred to HDV3, HDV5, and HDV7 sequences. The molecular clone of HDV (GenBank number M21012) used as positive control of PCR is included (C+). (B) Amino acid alignment of the C-terminal region of the HDV antigen. The last amino acids after the amber stop codon (*) are those present only in the HDV large antigen. Sequences from HDV genotype 1 and from the most divergent genotype 3 are shown. Sequence alignment and phylogenetic analysis were conducted using the Molecular Evolutionary Genetics Analysis version 7 software.

HCV infected without evidence of ongoing or past HBV infection, as shown by detection of HDV antibodies in 2 out of 160 patients who were HCV infected and who had undetectable markers of HBV infection and, for 1 patient, by low-level circulating HDV RNA. This suggests that HDV infection may occur in the absence of HBV infection and that HDV spread might take place in humans without HBV as a helper virus. Instead, HCV may have been responsible for HDV spread, although from the available clinical data, it is not possible to infer if HDV infection preceded, was simultaneous with, or occurred after HCV coinfection. Another possible explanation for this unusual pattern of HDV infection could be a coinfection with an occult HBV.[5] Although liver biopsy specimens of the patients were not available,

the absence of HBsAg and anti-HBc antibodies and the lack of detectability of serum HBV DNA by both qPCR and ddPCR are elements that can reasonably exclude an occult HBV infection.

Our results suggest transmission of HDV through HBV-unrelated viruses in humans. Several studies have shown that chronic HDV infection leads to more severe liver diseases than chronic HBV monoinfection. Hence, further studies addressing HDV presence and pathogenicity in large cohorts of patients with chronic HCV who are not infected with HBV may provide insights on alternative pathways used by HDV to spread with the help of "nonconventional" enveloped viruses[2] and insights on the origin of HDV, a highly significant human pathogen.

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