MAJOR ARTICLE

Cryptic Hepatitis B and E in Patients With Acute Hepatitis of Unknown Etiology

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Background. Up to 30% of acute viral hepatitis has no known etiology. To determine the disease etiology in patients with acute hepatitis of unknown etiology (HUE), serum specimens were obtained from 38 patients residing in the United Kingdom and Vietnam and from 26 healthy US blood donors. All specimens tested negative for known viral infections causing hepatitis, using commercially available serological and nucleic acid assays.

Methods. Specimens were processed by sequence-independent complementary DNA amplification and next-generation sequencing (NGS). Sufficient material for individual NGS libraries was obtained from 12 HUE cases and 26 blood donors; the remaining HUE cases were sequenced as a pool. Read mapping was done by targeted and de novo assembly.

Results. Sequences from hepatitis B virus (HBV) were detected in 7 individuals with HUE (58.3%) and the pooled library, and hepatitis E virus (HEV) was detected in 2 individuals with HUE (16.7%) and the pooled library. Both HEV-positive cases were coinfected with HBV. HBV sequences belonged to genotypes A, D, or G, and HEV sequences belonged to genotype 3. No known hepatotropic viruses were detected in the tested normal human sera.

Conclusions. NGS-based detection of HBV and HEV infections is more sensitive than using commercially available assays. HBV and HEV may be cryptically associated with HUE.

Keywords. liver diseases; next generation sequencing; shotgun library; hepatitis of unknown etiology.

Acute hepatitis of unknown origin (HUE) [1] indicates a disease associated with elevated aminotransferase and bilirubin levels in peripheral blood but not diagnosed on the basis of conventional serologic analysis to be due to infection by hepatitis viruses A through E. Other infectious agents, such as Epstein-Barr virus (EBV) [2], cytomegalovirus (CMV) [3], other herpesviruses [4], and parvovirus B19 [5], have also been reported to cause hepatitis symptoms. HUE may be acute or chronic [1]. Among acute cases of hepatitis, HUE comprised approximately 10% in the United States [6], 20% in Europe [7] and Japan [8], and >30% in South America [9] and Russia [10]. HUE was also observed in 5% of cases of chronic hepatitis in Japan and in 8% of acute and chronic cases in Eastern Europe [11].

Efforts to identify any etiological agents of HUE resulted in the discovery of GB virus A (GBV-A), GBV–B [12], GBV-C (hepatitis G virus [13]), TT virus (TTV) [14], SEN virus [15], NV-F [16], and KIs-V [17]. Association studies [18, 19] so far have not confirmed these viruses to be causes of HUE. The possibility remains that some cases of HUE may be due to occult infection by hepatitis B virus (HBV) [20] or hepatitis C virus (HCV).

The majority of approaches used to identify etiologic agents of HUE have relied on amplification aimed at conserved regions of the nonstructural viral proteins with degenerate primers [12, 17], representational difference analysis [14], or immunocapture [13]. Recent advances in high-throughput sequencing technologies provide new opportunities to studying an organism's metagenome and allow for open-ended detection of very low copy numbers of unique nucleic acids. Thus, next-generating sequencing (NGS), coupled with cell culture and the sequence-independent amplification approach

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to the library generation, was successful in identifying a novel canine hepacivirus [21] from dog liver and GBV-C from human brain [22]. Here, we report identifying HBV and hepatitis E virus (HEV) in many acute HUE cases by using modified complementary DNA (cDNA) amplification followed by NGS.

METHODS

Specimens

Serum specimens were collected from patients with acute hepatitis in Truro, United Kingdom (hereafter, "ENVD"; n = 32), and Vietnam (hereafter, "VNVD"; n = 6). The UK patients had presented with acute jaundice and were referred by their primary care physicians to the Jaundice Hotline service of the Royal Cornwall Hospital, with blood specimens collected within 72 hours of referral [23]. None of the patients received drug therapy in the 3 months before symptom onset, and none had clinical or laboratory evidence of autoimmune hepatitis. Noninfectious causes of jaundice, such as drug or alcohol toxicity, were excluded. Sera were tested for elevated alanine aminotransferase (ALT), alkaline phosphatase, and bilirubin levels and for anti-hepatitis A virus (HAV) immunoglobulin M (IgM), HBV surface antigen (HBsAg), HBV DNA, anti-HCV immunoglobulin G (IgG), HCV RNA, anti-HEV IgG and IgM (Wantai), IgG and IgM antibodies to EBV nuclear and viral capsid antigens, and anti-CMV IgM. All specimens tested negative for these viral markers. Sera from patients in Vietnam were collected during the acute phase and had elevated ALT and aspartate aminotransferase levels. All sera were negative for anti-HAV IgM, HAV RNA, HBsAg (BioRad), HBV DNA (polymerase chain reaction [PCR] sensitivity, 50 copies/mL), anti-HCV IgG, HCV RNA, and anti-HEV IgM (Genelabs Diagnostics); HEV RNA was tested by reverse transcription–PCR. Analyses of total anti-HBV core antibodies and anti-HBV surface antibody were also performed (BioRad). None of the patients were immunocompromised or positive for human immunodeficiency virus (HIV). Normal human sera from 26 US blood donors (BBI; now SeraCare) were used as controls. Patient data are presented in Table 1.

NGS

Total nucleic acid was extracted from 100 μL of serum (Total Nucleic Acid Kit, Roche, California) and recovered in a 20-μL final volume. Total nucleic acid was used for reverse transcription (SMART cDNA kit, Clontech, Mountain View, California). In the reverse transcription step, in addition to the SMART CDS Primer II A (5′–AAGCAGTGGTATCAACGCAGAGTACT(30) N-1N–3′), an alternative primer was used with the same 5′ end sequence but a random hexamer sequence in place of the T(30) at the 3′ end (5′–AAGCAGTGGTATCAACGGCAACAAN(6)–3′). The cDNA product was amplified (Advantage2 PCR, Clontech), and the resulting DNA was purified (AMPureXP, Agencourt), quantified, and used for generation of shotgun libraries for sequencing (TrueSeq DNA, Illumina, San Diego, California).

Table 1. Clinical Manifestation of Disease, Number of Reads Obtained, and Virus Identified Among Study Subjects

Patient[a]	Sex	Age, y	Clinical Diagnosis	ALT Level, IU/L	ALP Level, IU/L	AST Level, IU/L	Bilirubin Level, mg/mL	Jaundice	LFTR, wk	NGS Reads, No.[b]	Virus Identified
ENDV06AGH[c]	M	62	Acute hepatitis	2227	240	. . .	60	Yes	6	1 306 984	HBV
ENDV06LH	F	44	Acute hepatitis	1456	261	. . .	20	Yes	6	3 671 164	HBV
ENDV06GG[c]	M	62	Acute hepatitis	6357	311	. . .	241	Yes	. . .	4 660 965	HBV,[d] HEV
ENDV07CR	M	20	Acute hepatitis	1656	73	. . .	39	Yes	4	1 305 745	
ENDV07SG	F	69	Acute hepatitis	3807	. . .	. . .	57	Yes	9	844 273	
ENDV07JB	F	61	Acute hepatitis	1037	244	. . .	34	Yes	9	3 572 533	HBV, HEV
VNDV3	M	40	Acute hepatitis	600	. . .	500	. . .	. . .	. . .	236 464	
VNDV77	F	28	Acute hepatitis	510	. . .	500	. . .	. . .	. . .	343 802	HBV
VNDV85	F	42	Acute hepatitis	180	. . .	276	. . .	. . .	. . .	205 004	
VNDV87	F	40	Acute hepatitis	600	. . .	190	. . .	. . .	. . .	771 707	HBV
VNDV111	M	45	Acute hepatitis	500	. . .	450	. . .	. . .	. . .	122 461	HBV
VNDV114	F	53	Acute hepatitis	1140	. . .	597	. . .	. . .	. . .	213 486	
ENDV, pooled[e]	NA	NA	Acute hepatitis	NA	NA	NA	NA	NA	NA	674 665	HBV, HEV

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HEV, hepatitis E virus; LFTR, liver function time of recovery; NA, not applicable; NGS, next-generation sequence.

[a] All patients had individual shotgun libraries successfully prepared.

[b] Data were obtained using the Illumina platform and after removal of low-complexity sequences.

[c] The patient reported international travel.

[d] The complete genome was detected.

[e] Complementary DNA was pooled from 26 patients.

Sequence Data Analysis

To remove human sequences, all data were filtered using a Fast and Accurate Classification of Sequences (FACS) algorithm [24]. FACS builds a database, in which human k-mers are defined, stored, and used for data filtering, allowing for accurate and rapid classification of sequences as belonging to a reference sequence. The FACS algorithm uses small k-mers and has 99.8% sensitivity and 100% specificity when targeting the human genome, with an optimal k-mer size of 21 nucleotides. To filter low-complexity reads, the upper bound for Kolmogorov complexity [25] of each read was estimated; reads with a value lower than the cutoff were excluded. The complexity cutoff was calculated as the value that includes 99.67% of all reads of the same size that can be obtained from available viral genomes from GenBank. Filtered data were analyzed with CLC Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark), using a read mapping approach. The mapping was performed against a database containing complete viral genomes from *Flaviviridae* (n = 69), HAV (n = 50), HBV (n = 93), and HEV (n = 155). All hepatitis virus entries were retrieved from GenBank to include all known viral genotypes, subtypes, and variability. TTV, EBV, human herpesvirus 5, and CMV (GenBank NC_015783_TTV, AJ507799_EBV, NC_006273_HHV5, and X17403_CMV, respectively). The initial read mapping at 80% stringency should allow for detection of additional new variability. Reads matching known entities were collected. Reads that could not be mapped were also collected for further analysis by de novo assembly or remapping at varying stringency.

Genome Assemblies

Reads assigned by read mapping to known hepatitis viruses at 80% similarity were collected for analysis. To achieve an accurate contig assembly, the reads matching known entities were extracted and assembled de novo, as well with CLC Genomics Workbench 5.1. The generated consensi were used for reassembly, with all remaining sequences of undetermined genotype and new contigs were generated by increasing the homology stringency stepwise to 99%. All HBV reads of the same genotype from a patient were used in a mapping assembly, and the resulting consensi were extracted. Sequences that did not match the finalized contigs were used in BLASTn searches to establish their exact genetic origin.

Genotyping of Short HBV Reads

To unambiguously assign reads to the correct genotype, a distribution-free test based on the empirical distribution of intergenotype distances was designed and implemented. A set of 93 whole HBV genomes from genotype A (n = 17), B (n = 2), C (n = 18), D (n = 20), E (n = 5), F (n = 10), G (n = 4), H (n = 3), I (n = 3), and J (n = 1) was collected from GenBank. Multiple sequence alignment of the reference sequences was performed. Each read was aligned locally to each of the references and the genotype of the closest reference is chosen as a temporary match. Only reads with an aligned length of 100 nucleotides were used. The genomic region covered by the read is defined in the multiple sequence alignment. The Hamming distance among references is calculated for this particular region. For each read, a probability value was calculated as $P = (Y+1)/X$, where X is the total number of pairwise comparisons between sequences from the reference genotype and all others, and Y is the number of times that the observed genotype-to-read distance is greater than or equal to the between-genotype distances. The reads were assigned to the selected genotype if the P value was <.005, the level at which the null hypothesis that the read belongs to the distribution of intergenotype distances was rejected. All calculations were performed with MATLAB R2013b (The Math Works, Natick, Massachusetts).

RESULTS

HBV and HEV Sequences

Extraction of total nucleic acid from 12 sera obtained from the ENDV and VNDV HUE cases and 26 normal human sera yielded a sufficient amount of material to be sequenced as individual shotgun libraries. Total nucleic acids from 26 ENDV sera with insufficient yield were pooled to achieve the required target concentration and then used for NGS. Eight individual libraries were obtained from cases with particularly high ALT levels (>1000 IU/L). The average numbers of reads per library are listed in Table 1.

After filtering, the remaining sequences were mapped against sets of reference genomes. Reads obtained from 7 individually sequenced HUE samples matched HBV. The shotgun library generated from a pool of 26 total nucleic acids yielded 1 HBV read. Two of the individual libraries and the pool library yielded HEV sequences in addition to HBV (Table 1). Mapping of sequences from the normal human sera libraries to the same reference sets did not yield reads with homology to known hepatotropic viruses. A limitation of the study is that the healthy controls were not from the same geographical area as the subjects with HUE.

Whole-Genome HBV Sequences

From patient ENDV06GG, 856 reads were mapped to the reference set of 93 HBV genomes, with 319 reads mapping unambiguously ($P < .005$) to genotype G (HBV/G) and 45 mapping unambiguously to subtype A2 (HBV/A2; Figure 1*A*). The reads matching HBV/G were used to assemble a viral consensus sequence with a small 6-nucleotide gap (Figure 1*B*). Identification of an additional 179 reads matching this sequence at 99% homology allowed for assembly of a complete genome of HBV genotype G. This complete consensus genome contains the 36 nucleotide precore insertion characteristic of genotype G.

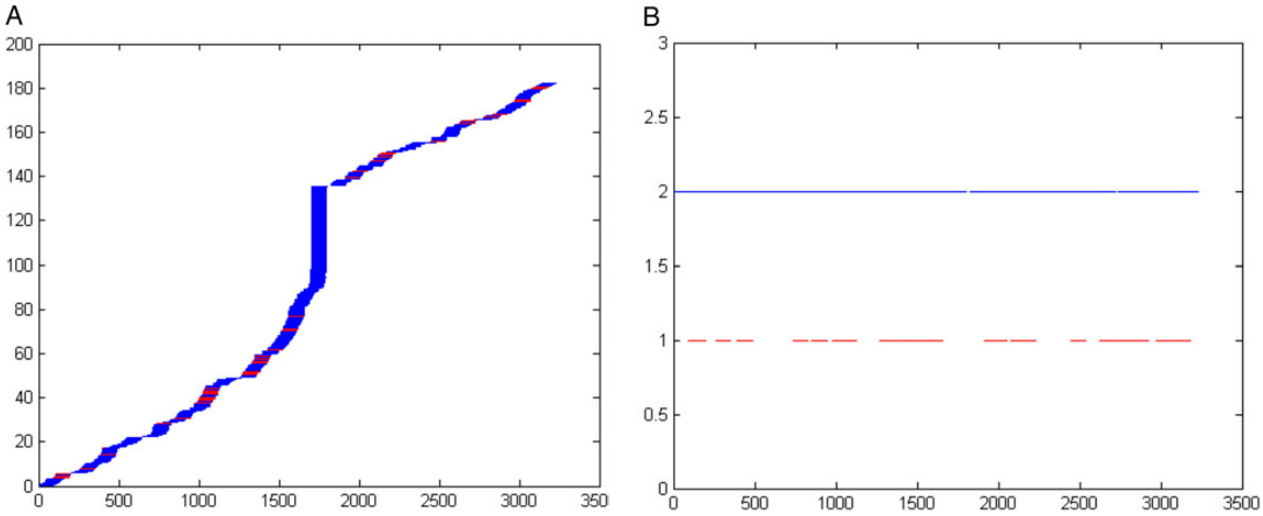

Figure 1. Filtered reads mapping to hepatitis B virus (HBV). *A*, HBV reads mapping unambiguously ($P < .005$) to HBV genotypes A (red) and G (blue). *B*, Unambiguous read assembled to individual HBV/A and HBV/G genomes.

The HBV/G consensus sequence was found to be nearly identical to that for certain HBV/G variants from GenBank (eg, GU563556), some of which were reportedly obtained from HIV-infected patients with occult HBV infection [26]. In addition to the 45 A2 reads, another 141 reads were recovered with 99% homology to genotype A2, but the resulting contig covered only 84.5% of the HBVA2 genome (Figure 1*B*); the best match (97%) for the A consensus was KP234051. The distributions of polymorphic sites along the HBV/G and HBV/A2 genomes are shown in Figure 2.

The HGV/G genomic region from patient ENDV06GG encodes the terminal protein and contains in-frame deletions at nucleotide positions 2656–2664 and 2733–2735, which do not affect the Tyr required for initiation of replication [27]. Both reads covering this region of the genome contain the deletion. It was not found in the HBV/A2 genome. Both the HBV/A2 and HBV/G sequences were examined for presence of precore/core mutations and known drug-resistance and vaccine-escape mutations. The HBV/G sequence contained mutations A1762T and A1764G, located in the base core promoter (BCP), and the G1896A mutation, which generates an in-frame stop codon in precore region [28]. All HBV/A2 reads covering the BCP and precore region contained wild-type sequences. The S gene of HBV/G and A2 encodes HBsAg belonging to subtype adw2. All HBV/G reads (n = 4) covering the "a" determinant contained G145R mutation [29], while in HBV/A2 only 1 of 5 reads contained G145R. No known drug-resistance mutations were detected in sequences of either genotype.

The remaining 172 HBV reads from patient ENDV06GG could not be genotyped confidently, because of their size and location within the HBV genome. Some reads contained terminal non-HBV sequences of 20–30 nucleotides, and 28 of those were mapped to various locations of the human genome, suggesting recombination among PCR products during amplification or HBV integration into the host genome. Additionally, 2 reads located around nucleotide position 1600 of the HBV genome could not be assigned to a particular HBV genotype. Both contained 8 polymorphic sites between nucleotide positions 1613–1638 matching HBV/G, in addition to 3 polymorphic sites between nucleotide positions 1570–1610 matching HBV/A, suggesting recombination between the 2 genotypes that may have occurred in vivo or during PCR. The complete genome of the genotype G HBV sequence ENDV06GG has the GenBank accession number KR230749.

HBV Sequences in Other Specimens

Fewer HBV reads were identified among the other 6 patients, for whom individual libraries were sequenced. These HBV sequences belonged to genotypes A, G, or D (Figure 3). In sera from 3 patients, HBV/G sequences were detected, with 2 of them also carrying HBV/A2 sequences. The pool of 26 sera produced 674 665 reads. One HBV sequence was found in that pool; however, HBV genotype could not be assigned. The genotypes observed are compatible with the geographical areas where the patients were sampled.

Partial HEV Sequences

Sera from 2 cases (ENDV06GG and ENDV07JB) contained reads of HEV genotype 3 (HEV/3) (Table 1). The 588 HEV/3 reads from ENDV06GG formed a contig of 578 nucleotides, and the 4 reads from ENDV07JB formed a contig of 185 nucleotides. Both were mapped to the 3′ end of the HEV genome between nucleotide positions 6550 and 7120. There was a single nucleotide difference between the consensus sequences from the 2 patients. The sequence had 94% homology to the closest

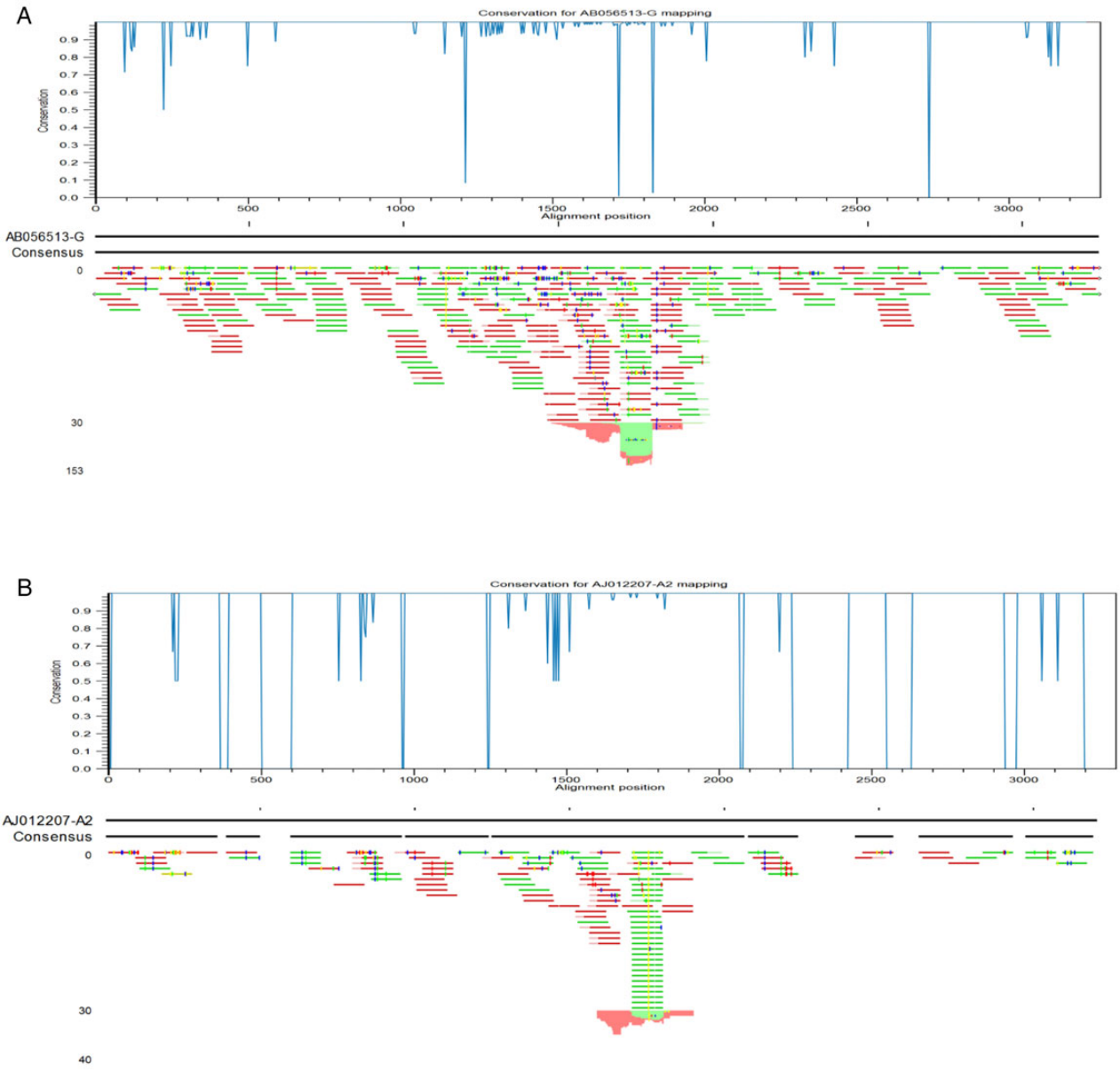

Figure 2. Variability per site chart and coverage display of the reads mapping to hepatitis B virus genotype G (HBV/G; average coverage, 15.29; *A*); and HBV/A2 (average coverage, 5.71; *B*).

HEV/3 sequence found in GenBank EU495148. Both sequences had few polymorphic sites. Seven reads of HEV/3 were also found in the pool of specimens from the other 26 ENDV cases.

DISCUSSION

Our findings support earlier observations [29] of seronegative HBV infections in >20% of cryptic hepatitis B cases, which are usually associated with viremia below the detection sensitivity of many commercially available nucleic acid tests. Previous studies of persons with seronegative HBV infections showed that HBV viremia may attain only about 102 HBV genome copies per mL. Such a level is below the detection limit of most assays for HBV DNA [30].

HBV/G reads were found in sera from 4 of 12 HUE cases, and in 3 of these 4, HBV/A2 reads were detected, as well. HBV/G is usually found in the presence of other genotypes, most frequently HBV/A2 [29], consistent with the hypothesis that HBV/G is not efficient in sustaining replication in the host unless associated with other genotypes [31]. All 4 patients had ALT levels exceeding 1000 IU/L, suggesting that HBV/G may play a role in the etiology of cryptic hepatitis. This suggestion is consistent with reports that mixed HBV/G and HBV/A infections are associated with severe hepatitis B and more-pronounced

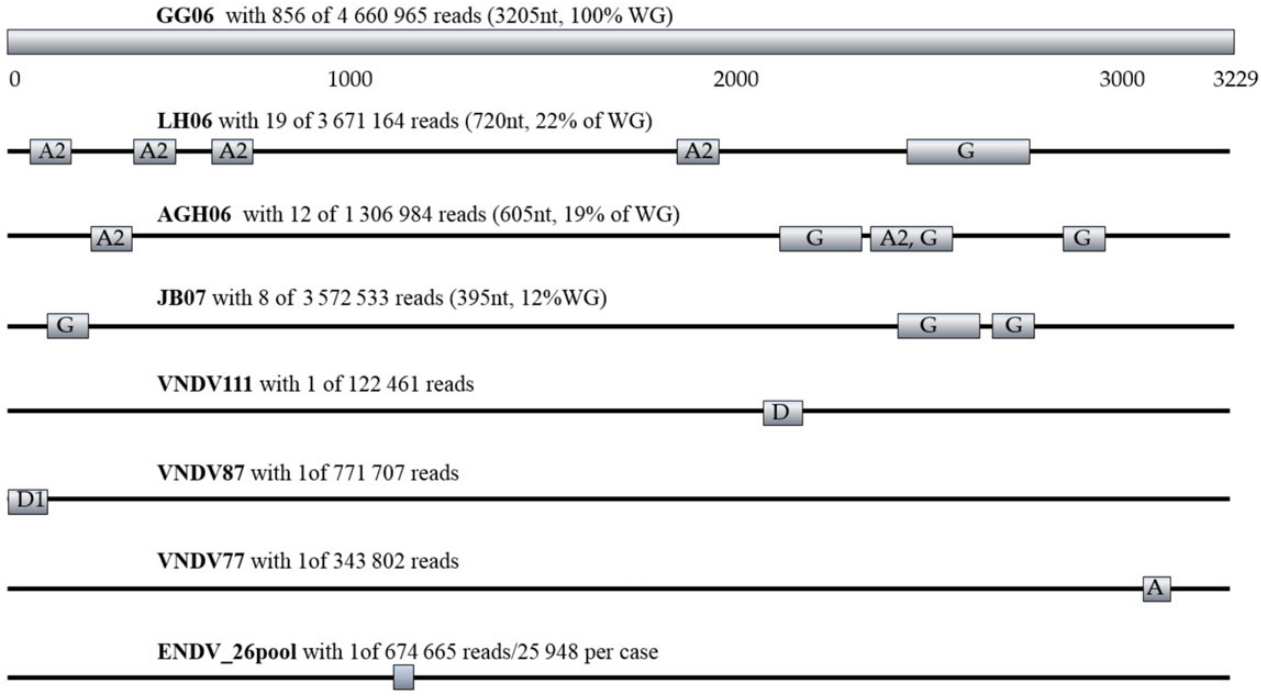

Figure 3. Hepatitis B virus (HBV) fragments found in serum specimens individually tested by next-generation sequencing (NGS). The complete genome isolate ENDVGG06 was used as genome map reference. All patients for whom HBV fragments were found are displayed on separate horizontal lines and labeled with their corresponding identification number, number of HBV reads, and total number of NGS reads. Values in parentheses denote the total combined length of the contigs found in a particular patient. Gray blocks with letters represent locations of HBV contigs and their genotype, when identified.

fibrosis [31, 32]. The molecular mechanism underlying the association of HBV/G with disease is not clear. Previous studies suggest that HBV/G may replicate in hepatocytes but at low level. In chimeric mice, coinfection with HBV/A or HBV/C significantly enhances its replication to such an extent that HBV/G eventually outgrows and takes over the coinfecting strain [32]. In mice, HBV/G takeover occurs more readily in those coinfected with HBV/A than with HBV/C [31]. HBV/G takeover was also observed in a patient with HBV/G and HBV/A coinfection [29]. In vitro studies show that, whereas the HBV/G core protein is functionally defective and the HBV/G core promoter is incapable of generating sufficient core protein, coinfection with HBV/A2 enhances HBV/G replication by providing core protein for packaging its genome, thereby facilitating egress of virions from the infected cells [33].

Although HBV/A2 coinfection has potential for facilitating HBV/G infection, HBV viremia was still low among all patients studied here. However, viremia levels in peripheral blood and liver may be poorly correlated [30]. This disparity may be more pronounced for HBV/G since it is secreted inefficiently from hepatocytes, resulting in retention of HBV products, thereby contributing to cellular distress. Intracellular accumulation of HBsAg may significantly affect cellular functions, potentially leading to hepatocyte stress [34]. Moreover, HBV/G was shown to be directly cytopathic in the presence of immunosuppression [31, 35], and despite a low replication rate, silent HBV infection was found to be associated with increased cytonecrotic activity and advanced liver disease [36]. Thus, high levels of biochemical markers of liver disease and low HBV viremia observed in the 4 patients studied here may be connected, possibly reflecting specific interactions of HBV/G with hepatocytes.

Enhanced virulence and serologically silent infections can also be explained by specific genetic modifications of the HBV genome. Indeed, inspection of the whole-genome sequences from patient ENDV06GG revealed several important mutations in the precore/core, S, and P genes. Mutations A1762T and A1764G in the BCP, together with G1896A generating the precore stop codon detected in the HBV/G sequence, are typically observed in this genotype [28, 37], supporting a role of HBV/G in association with hepatitis. All of these mutations affect expression of HBV e antigen (HBeAg) and may explain the lack of serological detection of this antigen and immune responses to both HBeAg and HBV core antigen. The S gene of HBV/G contained the G145R mutation in the "a" determinant. This mutation was also observed in 1 of 5 reads obtained from this genomic region in HBV/A2. The presence of G145R may explain the reduced sensitivity of conventional serologic assays to detect HBsAg in this patient. Additionally, the HBV/G genomic region encoding for the terminal protein contained a small in-frame deletion at nucleotide positions 2656–2664. Although the functional

significance of this deletion is not known, it can be hypothesized to lead to suppression of HBV replication, thus explaining the low viremia level in the patient.

Coinfection of HBV and HEV was found in 2 patients. One patient (ENDV06GG), from whom whole-genome sequences of HBV/G and HBV/A2 strains were obtained, showed high ALT and bilirubin levels (Table 1). After detection of HEV by NGS, testing of serum with an in-house PCR assay confirmed the presence of HEV RNA in this patient, while the other patient (ENDV07JB) remained negative for HEV by PCR. Although it is possible that HEV alone was responsible for liver disease in these 2 patients, the low viremia and lack of the detectable immune response are consistent with subclinical HEV infection [38]. Considering that co-occurrence of acute infections with 2 viruses, each having a different mode of transmission, is rare, detection of HEV in 2 of 7 patients with HBV infection suggests HEV superinfection of patients with preexisting, chronic HBV infection rather than coinfection with these 2 viruses. This suggestion is supported by earlier observations of acute HEV infection in patients with chronic hepatitis B [39]. In patients with underlying chronic liver disease, HEV superinfection can cause severe hepatic decompensation [40]. Long-term HBV DNA persistence for 10–30 years despite clinical and biological resolution of acute infection has been reported in approximately 55% of patients [28]. Persistence of HBV DNA in liver for 30 years without being detected in serum after well-documented acute hepatitis was also reported [39]. Thus, some of the patients with HUE in our study may have been chronically infected with HBV and manifested disease exacerbation following HEV superinfection.

In conclusion, detection of HBV and HEV sequence reads among patients with acute HUE suggests that they were cryptically infected with HBV and HEV. Human infections with HEV have been considered of no relevance to developed countries, but better tests and a growing body of evidence shows that they are of considerable global importance [41]. Whether such detection implies causation of acute disease remains to be confirmed as serum samples were collected only once from the patients, no liver samples were obtained, and no follow-up testing was done. The low HBV viremia levels and serologic absence of antigens or antibodies suggest that acute disease can be attributed, in some patients, to the unusual biological properties of HBV/G and to coinfection with HBV/G and HBV/A2. Detection of cryptic HBV infections with genotypes other than HBV/G indicates a potential role for different molecular mechanisms of HBV pathogenicity than those suggested here for HBV/G. It would be important to examine entire HBV genomes from more cases of HUE to understand the link between low HBV viremia levels and liver disease. For this purpose, NGS continues to be a method of choice, owing to its greater sensitivity to low-abundance DNA species. Finally, the etiology of approximately 40% of the HUE cases studied here remains unknown; whether HUE was due to HBV or HEV at even lower viremia levels or to other, yet undiscovered, pathogens requires further investigations.

Notes

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