Ⅲ．研究成果の刊行物一覧

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Research Letter

AIDS 2015, **29**:1717–1719

A cluster of rapid disease progressors upon primary HIV-1 infection shared a novel variant with mutations in the *p6^gag/pol^* and *pol/vif* genes

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Few studies have described the etiologic factors associated with rapid AIDS onset during primary HIV-1 infection. Our molecular epidemiological study identified a cluster of individuals infected with HIV-1 variants characterized by novel mutations in the *p6^gag/pol^* and *pol/vif* genes during 2011 and 2013 in Osaka, Japan. Individuals positive for the novel HIV-1 variant showed rapid disease progression, suggesting a role of viral mutations in the fostering of the clinical course of HIV-1 infection.

HIV-1 infection usually progresses to AIDS. Some HIV-1-infected individuals, known as long-term nonprogressors, maintain a $CD4^+$ T-cell count above 500 cells/μl and do not develop AIDS for more than 10 years after the primary HIV-1 infection (PHI) even without antiretroviral treatment [1]. Some long-term nonprogressors are categorized as elite controllers who can retain viral loads to undetectable levels [2]. In contrast, a small percentage of individuals develop AIDS within 2–3 years after a PHI [3,4]. Many factors, both viral and host, have been described as regulators of HIV-1 disease progression [5–7]. However, factors that foster HIV-1 disease progression remain to be clarified. Here, we describe a local epidemic involving an HIV-1 variant carrying novel mutations in *p6^gag/pol^* and *pol/vif* in individuals with PHI and rapid disease progression.

Since 1992, we have maintained a centralized confirmatory examination system at Osaka Prefectural Institute of Public Health for monitoring HIV-1 infection; this system involves a regional network of public health centers and primary care clinics that treat sexually transmitted infections. Serological and nucleic acid-based tests are performed, and HIV-1-positive specimens from the network are subjected to molecular epidemiological analysis of HIV-1 in Osaka prefecture based on HIV-1 C2V3 sequences of *env* [8].

In 2012, we encountered a case of a seronegative HIV-1 infection that refers to an individual negative for anti-HIV-1 antibody for prolonged period of time, although positive for p24 viral antigen and/or viral RNA; such cases have been rarely reported worldwide [9,10]. This case visited a hospital in the southern part of Osaka prefecture and was diagnosed with PHI via nucleic acid-based tests. However, it required more than 8 months until the results of western blot become fully positive for anti-HIV-1 antibodies (Fig. 1a). The virus infecting this individual clustered genetically with viruses isolated from six other individuals with PHI; these seven cases were mostly from the same district and were reported during 2011–2012. Those viruses were of subtype B with a putative CCR5 tropism based on the geno2pheno algorithm [11]. Interestingly, the clinical features of those PHIs were similar in that they involved extremely high plasma viral loads (>10^7 copies/ml) and low $CD4^+$ T-cell counts less than 200 cells/μl (range, 64–184). Of the seven individuals, four initiated antiretroviral therapy immediately after diagnosis of HIV-1 infection due to severe clinical symptoms; two of these four cases progressed to AIDS despite the early phase of HIV-1 infection.

We investigated whether the viruses infecting these seven individuals with PHI shared any genetic features. Population sequencing of the HIV-1 *gag-pol* gene (Methods, Supplemental Digital Content 1, http://links.lww.com/QAD/A730), which is routinely used for monitoring antiretroviral drug resistance, demonstrated that these viruses shared the following genetic signatures: a 15-nucleotide insertion into *p6^gag/pol^*, the *gag-pol* transframe region, resulting in duplication of the N-QSRPE-C pentapeptidic sequence in the *p6^gag^* open reading frame and of N-EQTRA-C in the *p6^pol^* open reading frame (Fig. 1b) and two substitution mutations in the *pol/vif* overlapping region; one mutation changed the *pol* stop codon into a Gln-coding codon (TAG to CAG), whereas the second mutation, located 12 nucleotides downstream of the other mutation, generated both an aberrant stop codon in the *pol* gene (AAG to TAG) and a Lys-to-Asn codon replacement (AAA to AAT) in the 22nd amino acid of the Vif protein (Fig. 1c). The insertion mutation was located immediately upstream of the $p6^{Gag}$ PTAP motif, which is known to support viral budding [12,13]. The substitution mutations should result in the addition of an aberrant tetrapeptide N-QNME-C at the Pol carboxy terminus, namely integrase. The Vif amino acid alteration was positioned near the interface between Vif and core binding factor β; therefore, this change might affect Vif-APOBEC3G interactions [14,15]. Although insertional mutations in *p6^gag^* are described frequently [16,17], the N-QSRPE-C duplication is very rare. Moreover, an HIV-1 variant carrying both *p6^gag/pol^* and *pol/vif* mutations has not, to our

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(a)

Patient ID	HIV risk factor	Samping time month/year	pVL copies/mL	CD4 cell count cells/mm^3	WB status	ART
11–65	MSM	08/2011	>10,000,000	136	±	–
		09/2011	1,800,000	70	ND	+
12–27*	unknown	03/2012	>10,000,000	241	–	–
		07/2012	1,200,000	64	–	–
		09/2012	15,000	198	–	+
		11/2012	1,100	221	±	+
		01/2013	96	264	+	+
12–52	MSM	06/2012	>10,000,000	184	–	–
		09/2012	99,000	530	+	–
		07/2013	11,000	410	+	–
12–86	MSM	10/2012	>10,000,000	117	–	–
		11/2012	9,300,000	334	±	–
		12/2012	9,700	651	+	+
12–1767	MSM	07/2012	ND	ND	–	–
		08/2012	> 10,000,000	79	±	+

(b)

```
                p1  |  p6
                  450
AA Pol         S  S  E  Q  T  R  A  -  -  -  -  -  N  S  P  T  R  R  E  L  Q  V  W
AA Gag       F  L  Q  S  R  P  E  -  -  -  -  -  P  T  A  P  P  E  E  S  F  R  S  G
HXB2        TTTCTTCAGAGCAGACCAGAG--------------CCAACAGCCCCACCAGAAGAGAGCTTCAGGTCTGGG

Novel HIV-1 TTTCTTCAGAGCAGACCAGAGCAGAGCAGACCAGAGCCAACAGCCCCACCAGAGGAGAGCTTCAGGTTTGGG
AA Gag       F  L  Q  S  R  P  E  Q  S  R  P  E  P  T  A  P  P  E  E  S  F  R  F  G
AA Pol         S  S  E  Q  T  R  A  E  Q  T  R  A  N  S  P  T  R  G  E  L  Q  V  W
```

(c)

```
AA Vif        M  E  N  R  W  Q  V  M  I  V  W  Q  V  D  R  M  R  I  R  T  W  K  S
AA IN       Y  G  K  Q  M  A  G  D  D  C  V  A  S  R  Q  D  E  D  *
HXB2        TATGGAAAACAGATGGCAGGTGATGATTGTGTGGCAAGTAGACAGGATGAGGATTAGAACATGGAAAAGT

Novel HIV-1 TATGGAAAACAGATGGCAGGTGATGATTGTGTGGCAAGTAGACAGGATGAGGATCAGAACATGGAATAGT
AA IN       Y  G  K  Q  M  A  G  D  D  C  V  A  S  R  Q  D  E  D  Q  N  M  E  *
AA Vif        M  E  N  R  W  Q  V  M  I  V  W  Q  V  D  R  M  R  I  R  T  W  N  S
```

Fig. 1. Clinical and genetic features of the novel HIV-1 variants. (a) Serological examination data were available from five patients infected with the novel HIV-1 variants; one case involved a seronegative infection (*), and two cases involved rapid onset of AIDS (underlined). High plasma viral loads and low CD4 counts were observed before or at seroconversion. pVL, plasma viral load; ART, antiretroviral therapy. Western blot status: –, negative; ±, indeterminate; +, positive; ND, not determined. (b and c) Genetic signatures of the novel HIV-1 variants. HXB2 is a molecular clone representing the standard HIV-1 clade B. (b) A duplication within *p6*$^{gag/pol}$ resulted in a 5-amino acid repeat (underlined), and (c) substitution mutations within *pol/vif* resulted in the addition of four amino acids at the integrase C-terminus and an amino acid substitution in Vif (underlined).

knowledge, been previously reported. These HIV-1 variants were not predicted to have major mutations conferring resistance to any of the antiretroviral drugs.

Careful re-evaluation of our regional molecular surveillance data revealed that this novel HIV-1 variant was present in another 10 specimens examined between 2011 and 2013; these variants genetically clustered with the seven variants described above. Interestingly, HIV-1 variants bearing similar substitution mutations in the integrase were also detected in eight specimens collected between 2009 and 2014. Of these eight variants, three were non-B subtypes that were located separately in the genetic phylogeny (Figure, Supplemental Digital Content 2, http://links.lww.com/QAD/A730). Most of the novel HIV-1 variants and closely related variants were detected in specimens collected from MSM and were from the same geographical area in Osaka. The novel HIV-1 variant described here was not captured before 2011.

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Molecular epidemiology and clinical indicators of the seven PHI cases, including the two AIDS cases, suggested that these individuals might have been subject to rapid HIV-1 disease progression due to the infection of HIV-1 variants bearing a unique set of mutations. To our knowledge, this is the first study describing a link between the rapidness of HIV-1 disease progression and the genetic features of HIV-1 isolates from a local epidemic. Molecular epidemiology indicated that each unique genetic mutation in these novel HIV-1 variants probably emerged independently in recent years and then converged via recombination in the local community. Clinical and virological details should provide insights into the pathogenesis of HIV-1 infection. Previously, it was noted that the seronegative HIV-1 infection could be observed upon PHI in which a massive destruction of host immunity took place quickly [9]. This pathophysiology is closely related to rapid disease progression. Seronegative HIV-1 infection is problematic because rapid HIV-antibody tests are still widely used as primary screening tests at voluntary counseling and testing sites. Consequently, we would like to call attention to these novel HIV-1 variants.

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This study was approved by the ethical review board of Osaka Prefectural Institute of Public Health (approval numbers:0703-06, 1310-08 and 1409-05).

Author contributions: H.M., T.K. and J.K. conceived the study. M.M., K.U. and M.K. provided clinical data. H.M., Y.K. and T.K. contributed to laboratory work. H.M., Y.K., T.K. and J.K. wrote the article. All authors have read and approved the final article as submitted to AIDS.

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Conflicts of interest

There are no conflicts of interest.

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Supplemental Digital Content

Methods. SDC 1.

Serological and nucleic acid examination:

Serum samples positive in HIV-screening tests were sent to Osaka Prefectural Institute of Public Health, and HIV-1 infection was confirmed via western blot (WB) assays using LAV BLOT 1 (Bio-Rad, Tokyo, Japan). Samples negative and/or indeterminate on WB assays were subjected to in-house real-time PCR assays using KK-TaqMan probes [S1] or to COBAS TaqMan assays at a commercial laboratory.

Genetic analysis:

Viral RNA was extracted from serum samples, and reverse transcription (RT) and nested PCR were performed as previously described [8]. The primer sequences were as follows: SK38 5'-ATA ATC CAC CTA TCC CAG TAG GAG AAA T-3' and RT20 5'-CTG CCA GTT CTA GCT CTG CTT C-3' for RT and the initial PCR, PR05 5'-AGA CAG GYT AAT TTT TTA GGG A-3' and RT4L 5'-TAC TTC TGT TAG TGC TTT GGT TCC-3' for nested PCR amplification of the *gag*-RT region, IN-Fout 5'-CAG ACT CAC AAT ATG CAT TAG G-3' and IN-Rout 5'-CCT GTA TGC AGA CCC CAA TAT G-3' for RT and the initial PCR, and IN-Fin 5'-CTG GCA TGG GTA CCA GCA CAC AA-3' and IN-Rin 5'-CCT AGT GGG ATG TGT ACT TCT GAA CTT A-3' for nested PCR amplification of the integrase region. Nucleic acid sequencing was performed via the dideoxy method with BigDye terminator (Applied BioSystems, Tokyo, Japan). Nucleic acid sequences were determined with a ABI 3130 Genetic

Analyzer (Applied BioSystems) via direct sequencing. The nucleic acid and amino acid sequences were compared with those of the reference strain HXB2 (accession No. K03455). A phylogenetic tree was constructed using the neighbor-joining method in MEGA 5 software [S2].

Nucleotide sequence accession numbers:

The novel HIV-1 *p6* and integrase sequences are available in GenBank under the following accession numbers:

LC033998-LC034020; LC033853-LC033869; AB870487; AB870497; AB870499; AB870503; AB870516; AB870509; AB870512; AB870525

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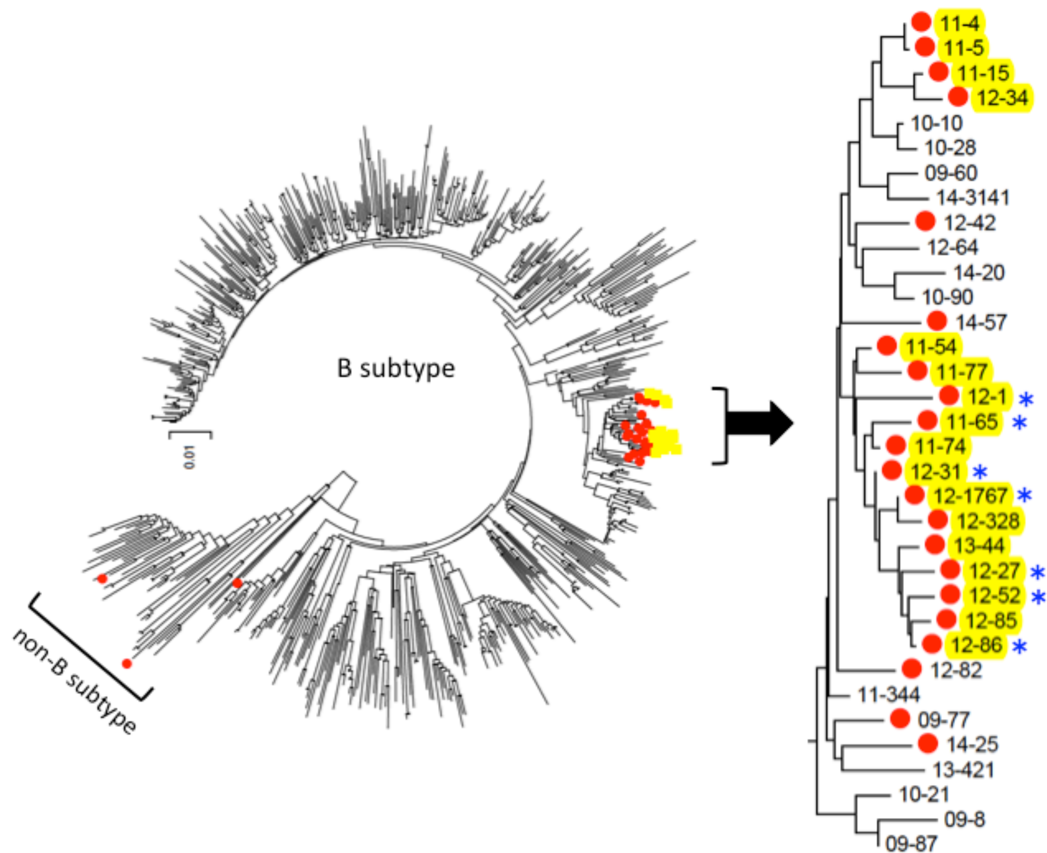

Figure. SDC 2. Phylogenetic analysis of the HIV-1 integrase sequences obtained during 2008 – 2014 in the regional surveillance in Osaka, Japan (n = 646). The novel HIV-1 variants form a distinct cluster in this phylogenetic tree (■). HIV-1 isolates carrying an integrase mutation identical to that in the novel HIV-1 variants are indicated by red (●). Three red isolates, which are separate (lower left) from the major cluster, are of non-B subtypes. Asterisks (*) indicate HIV-1 detected in patients with primary HIV-1 infection defined as negative or indeterminate on WB, but positive for HIV-1 RNA.

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Identification of Novel Recombinant Forms of Hepatitis B Virus Generated from Genotypes Ae and G in HIV-1-Positive Japanese Men Who Have Sex with Men

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Abstract

The rare hepatitis B virus (HBV) genotype G (HBV/G) coinfects HIV-1-positive individuals along with HBV/A and generates recombinants. However, the circulation of HBV A/G recombinants remains poorly understood. This molecular epidemiologic study examined HBV A/G recombinants in Japanese HIV-1-positive men who have sex with men (MSM). Initially, blood specimens submitted for confirmatory tests of HIV infection in Osaka and Tokyo, Japan, from 2006 to 2013 were examined for HIV-1, and HIV-1-positive specimens were screened for HBV. Among 817 specimens from HIV-1-positive individuals, HBsAg was detected in 59 specimens; of these, HBV/Ae (alternatively A2), a subgenotype of HBV/A prevalent in Europe and North America, was identified in 70.2%, HBV/C in 17.5%, and HBV/G in 10.5%, and HBV/E in 1.8% according to the core gene sequence. The full-length genome analysis of HBV was performed on HBV/G-positive specimens because some HBV A/G recombinants were historically overlooked by genotyping based on a partial genome analysis. It revealed that five of the specimens contained novel Ae/G recombinants, the core gene of which had a high sequence similarity to HBV/G. Detailed analyses showed that novel recombinants were coinfected with HBV/Ae in a recombinant-dominant fashion. No major drug-resistant mutations were found in the newly identified HBV Ae/G recombinants. Some of the individuals asymptomatically coinfected with HIV/HBV suffered mild liver injury. This study demonstrated that novel Ae/G HBV recombinants were identified in Japanese HIV-1-positive MSM. The pathogenicity of novel HBV Ae/G recombinants should be examined in a future longitudinal study. Surveillance of such viruses in HIV-1-positive individuals should be emphasized.

Introduction

Hepatitis B virus (HBV) is a member of the hepadnavirus family, which is associated with acute and chronic hepatitis and hepatocellular carcinoma. These conditions cause considerable morbidity and mortality.[1] According to the World Health Organization, over 350 million people worldwide are chronically infected with HBV (WHO; www.who.int/mediacentre/factsheets/fs204/en/). HBV has a partially double-stranded DNA genome, which is approximately 3.2 kbp in size and encodes four overlapping open reading frames: P, pre-S/S, pre-C/C, and X.[2] HBV is highly diverse, with a mutation rate estimated at $1\text{–}5 \times 10^{-5}$ nucleotide substitutions per site per year.[3,4] This rate is intermediate between that of DNA and RNA viruses because the HBV genome replicates through an RNA intermediate using a virus-encoded reverse transcriptase that lacks a proofreading function.[4]

HBV is classified into 10 genetic groups, termed HBV/A to J, based on an intergroup divergence of >8%.[3–9] Each genotype was divided into subgenotypes. The genotypes of HBV show a distinct geographic distribution[10,11] and are associated with different clinical outcomes, responses to treatment with interferon or nucleotide analogues, and rates of fulminant hepatitis.[12–21]

Historically, 96.9% of HBV isolates from Japanese individuals with chronic hepatitis B belong to genotypes B or C[14]; however, since 2000, HBV/A has spread in urban areas through homosexual intercourse.[18,21–23] The positivity rate for HBV infection in Japanese HIV-1-infected individuals is 8.9%, and

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half of HBV-infected individuals harbor the genotype A virus.[24] A previous report showed that up to 90% of HBV isolates obtained from HIV-1-infected individuals were genotype A.[25] Our previous surveillance study focusing on high-risk populations that attended primary sexually transmitted infection clinics in Osaka revealed that 60% of the HBV genotypes infecting HIV-1-positive individuals were Ae (alternatively, A2), a distinct genetic cluster within HBV genotype A that was distributed in Europe and North America.[26,27] In that study, we noticed that three HIV-1-positive individuals were coinfected with HBV/G, a rarely identified genotype of HBV.

Genotype G is an unusual variant of HBV and little is known about its epidemiology, natural history, and clinical data.[28] In 2000, a unique HBV isolate harboring a 36-base pair insertion in the core region was identified in France; this was the first isolate of genotype G.[6] The S gene of HBV/G is highly homologous (94.6–97.5%) with that of HBV/A at the nucleotide (nt) level.[29] Subsequently, HBV/G was identified in the United States,[30,31] Mexico,[32] Germany,[33] Canada,[28] and Brazil.[34] A previous study estimated the prevalence of HBV/G in these areas to be 1–5%. HBV/G infection occurred predominantly in males (92%) and was primarily associated with homosexuals.[28] Indeed, HBV/G-positive individuals were coinfected with HBV/A or a recombinant genotype A/G virus,[28] which is consistent with other reports, including our own.[24,26]

Recently, a number of reports documented homologous recombination between different HBV genotypes.[1] A concern is how frequently such recombinations occur, and whether the pathogenicity of recombinant HBVs differs from that of other HBV genotypes. Because HBV/G coinfects with HBV/A, genetic recombination between these genotypes is possible. Indeed, five A/G recombinants have been identified to date: two in the United States (AB056516[30,35] and JQ707426[36]), two in Canada (EU83389 and EU83390[28]), and one in Brazil (EF464099[34]).

Until now, genotyping of HBV has been based on only a part of the viral genome. Importantly, analysis of the complete genome sequences of HBV isolates revealed that a viral clone previously classified as genotype G was actually an A/G recombinant.[30,35] In our previous study, our classification of the HBV genotype was not based on the full viral genome sequence, raising concerns that some A/G recombinant forms might have been overlooked. Thus, we revisited the clinical specimens to ascertain whether any A/G recombinant forms of HBV were detectable upon full genome analysis.

Materials and Methods

Specimens and serological diagnosis

Specimens found to be HIV-1 positive in the confirmatory tests were subjected to screening for the HBs antigen (Ag). A total of 817 specimens, 813 from Osaka and four from Tokyo, collected either at health care centers or medical institutions between 2006 and 2013, were analyzed.

An HIV serodiagnostic screen was performed using the Genedia HIV-1/2 mix particle agglutination (PA) anti-HIV assay method (Fujirebio, Inc., Tokyo, Japan). Confirmatory tests for HIV-1 infection were performed using LAV BLOT 1 (Fujirebio). Serological tests for HBV were performed using the Espline HBsAg kit and the SERODIA-anti-HBs PA or Espline HBsAb-N kits, which detect anti-HBs antibodies (Ab) (Fujirebio). Serological tests for HBV were also performed using the Mycell anti-rHBc diagnostic reagent, which detects anti-HBcAb (Institute of Immunology Co. Ltd., Tokyo, Japan). Serodiagnosis of *Treponema pallidum* (TP) infection was performed using the Serodia TP-PA test (Fujirebio).

Biochemistry

Plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and gamma-glutamyl transpeptidase (γ-GTP) were measured. All specimens were diluted 2- to 20-fold in phosphate-buffered saline (PBS) prior to measurement.

Viral load tests

The HIV-1 viral load was measured using AmpliPrep/Cobas TaqMan HIV-1 v2.0 (Roche Diagnostics, Manheim, Germany) or by real-time polymerase chain reaction (PCR) according to the method described by Kondo *et al.*[37] The HBV viral load was measured using the COBAS AmpliPrep/Cobas TaqMan HBV Test v2.0 (Roche).

Analysis of the HIV-1 genome

The HIV-1 genome was analyzed as described previously.[38] Briefly, the *env* C2-V3 and *pol* regions of the HIV-1 genome were analyzed and the HIV-1 subtype was determined by phylogenetic analysis using GENETYX-MAC Ver. 14 (GENETYX, Tokyo, Japan). HIV drug resistance was determined using the HIVdb Program in the HIV drug resistance database (http://sierra2.stanford.edu/sierra/servlet/JSierra).

Analysis of the HBV genome

DNA was extracted from HBsAg-positive sera using a QIAamp UltraSens Virus Kit (QIAGEN, Dusseldorf, Germany), and the HBV DNA was PCR amplified. The primers were WA-L, WA-R, HBVnext, and AS2330 (WA-L, WA-R, and AS2330 have been described previously) (Table 1).[39,40] PCR products were electrophoresed in 1% agarose gels. The DNA bands were extracted and sequenced using the BigDye Terminator kit (Applied Biosystems, Foster, CA) and the following primers: WA-L, WA-R, HBVnext, AS2330, FA1-L, FA2-L, FA3-L, FA4-L, FA1-R, FA2-R, FA3-R, FA4-R, and 1868 (Table 1).[39,40] Nucleic acid sequencing was performed in a ABI3130 automated sequencer (Applied Biosystems). Nucleic acid sequence analysis was performed using BLAST, CLUSTAL W (DDBJ: DNA Data Bank of Japan), GENETYX-MAC Ver. 14 (GENETYX, Tokyo, Japan), Molecular Evolutionary Genetics Analysis (MEGA) 5.2.2 and the Oxford HBV Automated Subtyping Tool (http://jose.med.kuleuven.be/genotypetool/html/indexhbv.html), and the method developed by de Oliveira *et al.*[41] Neighbor-joining and Kimura two-parameter models were employed to analyze with MEGA software. A gene-by-gene analysis was performed using MEGA and Clustal W software.

HBV genotype-specific PCR and cloning of PCR products

Nested PCR was performed to clarify coinfection by distinct HBV genotypes. Briefly, the initial PCR was performed using the HBVALL9/HBVALL12 primer set to generate products of 1,615 and 1,651 bp, representing genotypes Ae

TABLE 1. PRIMERS USED IN THIS STUDY

Name	Nucleotide sequence (5′–3′)	Position[a]	Orientation	Product size (bp)	Reference
WA-L	ACTGTTCAAGCCTCCAAGCTGTGC	1859–1882	Sense	3,191	Zhang et al.[39]
WA-R	AGCAAAAAGTTGCATGGTGCTGGT	1828–1805	Antisense		Zhang et al.[39]
HBVnevt	TCATCTGCCGGACCGTGTGCACTT	1563–1586	Sense	765	This study
AS 2330	GTGTTGATAAGATAGGGGCATTT GGTGG	2327–2300	Antisense		Vitale et al.[40]
HBVALLF9	GTTTACTAGTGCCATTTGTTCAG	675–697	Sense	1,294 (Ae) or 1,330 (G)	This study
HBVALLR10	AAGTCAGAAGGCAAAAACGAGAG	1968–1946	Antisense		This study
HBVAEF1	GGATCATATTGTACAAAAGATC	915–936	Sense	568 (Ae, G, Ae/G)	This study
HBVGF2	AGAACACATCACACAGAAAATT	915–936	Sense		This study
HBVAER3	GAGTCCCAAGCGGCCCCGA	1482–1464	Antisense		This study
HBVGR4	GAGCCCCAAACGGCCCCGG	1482–1464	Antisense		This study
HBVALLF9	GTTTACTAGTGCCATTTGTTCAG	675–697	Sense	1,615 (Ae) or 1,651 (G)	This study
HBVALLR12	GGAGGAGTGCGAATCCACACTCC	2289–2267	Antisense		This study
HBVAEF5	GCATGGAGACCACCGTGAACGCCCA	1606–1630	Sense	387 (Ae)	This study
HBVAER7	TAGGAGATCTCTGACGGAAGGAAAG	1992–1968	Antisense		This study
HBVGF6	ACATGGAAACCGCCATGAACACCTC	1606–1630	Sense	423 (G)	This study
HBVGR8	GAGAAGATCACGAACAGACGGGAAA	1992–1968	Antisense		This study
HBVAEF14	GTCAATCTCCGCGAGGACTG	118–137	Sense	1,365	This study
HBVAER3	GAGTCCCAAGCGGCCCCGA	1482–1464	Antisense		This study
Sequencing primers					
FA1-L	TTTCACCTCTGCCTAATCATCTC	1823–1845	Sense		Zhang et al.[39]
FA2-L	GCGTCGCAGAAGATCTCAAT	2419–2438	Sense		Zhang et al.[39]
FA3-L	CTGCTGGTGGCTCCAGTT	57–74	Sense		Zhang et al.[39]
FA4-L	GTATTGGGGGCCAAGTCTGT	751–770	Sense		Zhang et al.[39]
FA1-R	TCTTGTTCCCAAGAATATGGTG	2845–2824	Antisense		Zhang et al.[39]
FA2-R	TTGAGAGAAGTCCACCACGAG	273–253	Antisense		Zhang et al.[39]
FA3-R	GCCTTGTAAGTTGGCGAGAA	1115–1096	Antisense		Zhang et al.[39]
FA4-R	AAAAAGTTGCATGGTGCTG	1825–1807	Antisense		Zhang et al.[39]
1868	GCCTCCAAGCTGTGCCTTGGGTGG	1868–1891	Sense		Vitale et al.[40]

[a]According to the AB014370 coordinate.

and G, respectively (Table 1). The second PCR was performed using the HBVAEF5/HBVAER7 or HBVGF6/HBVGR8 primer sets, yielding products of 387 bp or 423 bp corresponding to HBV genotypes Ae or G, respectively (Table 1). The PCR products were confirmed by sequencing. Additionally, nested PCR primers were designed to amplify genomic DNA specific for HBV/Ae, HBV/G, and Ae/G recombinants (see Table 1).

The initial PCR was performed using the HBVALL9/HBVALL10 primer set, which amplifies all HBV genotypes. The second PCR was performed using the HBVAEF1/HBVAER3, HBVGF2/HBVGR4, or HBVAEF1/HBVGR4 primer sets, which yielded amplicons only when the template contained the Ae, G, or Ae/G genotypes, respectively. To determine the ratio of coinfecting HBV genotypes, the PCR products primed by HBVALL9/HBVALL10 were cloned into the pTAC-1 vector and sequenced (BioDynamics Laboratory, Inc., Tokyo, Japan). The amplicon was scored HBV genotype G-derived when it contained the 36-bp signature sequence unique to HBV genotype G. To analyze the genome sequence of HBV/Ae that was coinfected with the HBV Ae/G recombinant, the PCR products primed by AEF14/AER3 were sequenced.

Ethical considerations

This study was approved by the Ethical Review Board of Osaka Prefectural Institute of Public Health (approval numbers 0703-06, 0810-4, and 0810-5-2).

Results

Initial screening of HIV-1-positive individuals for HBV genotype G

We examined 817 blood samples collected from HIV-1-positive individuals from 2006 to 2013. The primary screen employing an immunochromatographic test for HBs Ag identified 59 positive specimens. HBV DNA was successfully amplified from 57 of these specimens by PCR targeting the core region of the viral genome. Sequencing analysis revealed that the most common HBV genotype was Ae (40/57 specimens, 70.2%), followed by C (10/57 specimens, 17.5%), G (6/57 specimens, 10.5%), and E (1/57 specimens, 1.8%). We focused our attention on six specimens that were positive for HBV genotype G, since this genotype is rarely detected worldwide. PCR successfully amplified the complete HBV genome from five of the specimens. These were named Os/JP/2008, Tk/JP/2009, Os/JP/2010, Os/JP/2011, and Os/JP/2013. All five individuals infected with HBV/G were men who have sex with men (MSM).

Identification of novel Ae/G recombinants of HBV

Phylogenetic analysis of the full length viral genome sequences revealed that Os/JP/2011 and Os/JP/2013 formed a distinct cluster under the genotype A group, and Os/JP/2008, Tk/JP/2009, and Os/JP/2010 formed a distinct cluster under the genotype G group (Fig. 1). However, we noticed that previously isolated Ae/G recombinants positioned in the

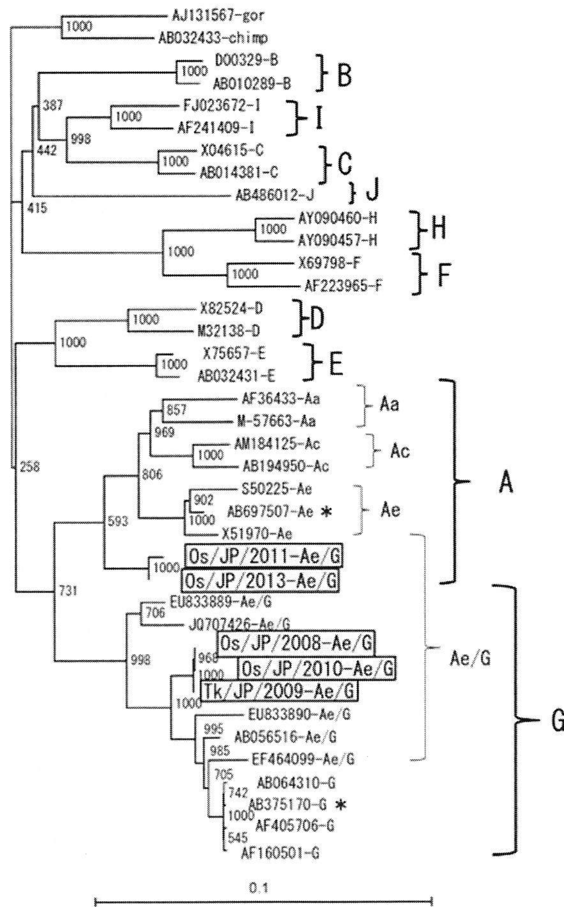

FIG. 1. Phylogenetic analysis of novel hepatitis B virus (HBV) A/G recombinants and comparison with representative strains from each HBV genogroup. Genetic distances were estimated using the full length genome sequences of HBV strains and the Kimura two-parameter matrix method. Phylogenetic trees were drawn using the neighbor-joining method. Accession numbers and clone names are shown on each branch. Bootstrap values are shown along with each main branch. A scale bar indicates the degree of nucleotide divergence. The novel Ae/G recombinants isolated in this study are *boxed*. Viral strains marked with an *asterisk* are reference strains representing genotypes Ae and G (Fig. 2).

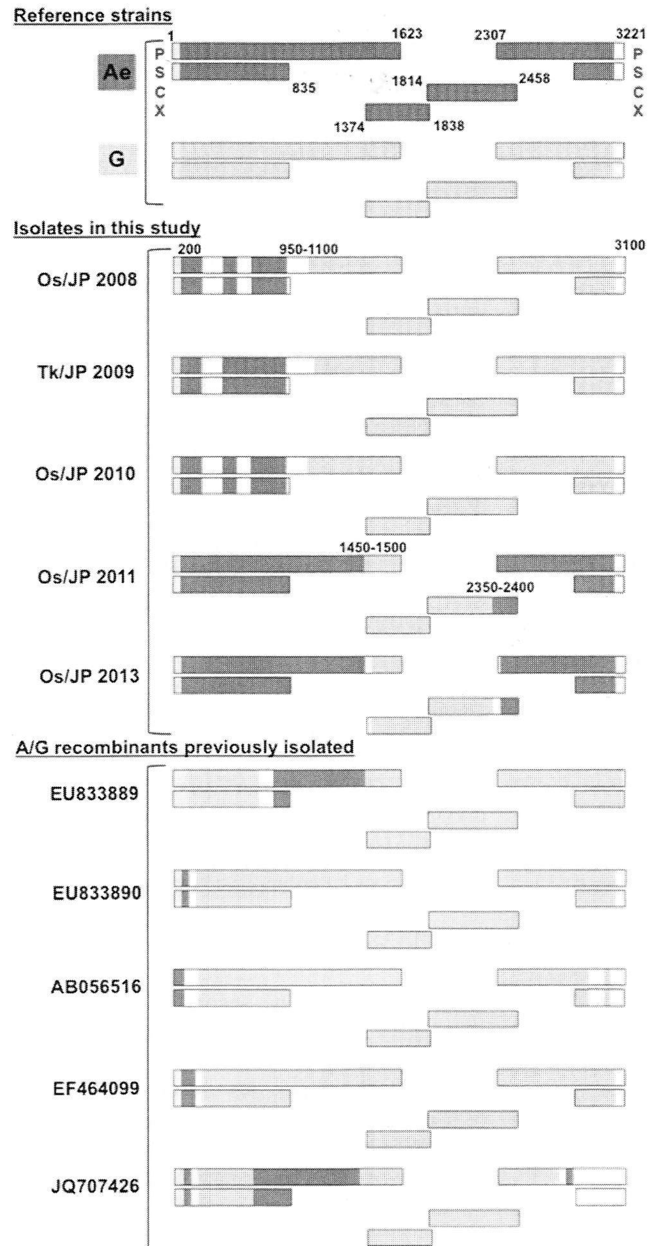

FIG. 2. Genetic structures of the novel HBV Ae/G recombinants isolated in this study. The genomes of HBV/Ae and HBV/G are shown in *dark gray* and *gray*, respectively. Bootscan analysis was performed with a window size of 400 and a step size of 50. The representative strains of Ae and G are AB697507 and AB375170, respectively (*asterisks* in Fig. 1). The approximate locations of the recombination breakpoints are shown. All five previously isolated A/G recombinants are shown for reference. P, polymerase gene; S, surface gene; C, core gene; X, the X gene.

neighboring branches. Genetic structure analysis and gene-by-gene analysis revealed that all the viruses initially judged as genotype G according to C gene sequences were A/G recombinants (Fig. 2 and Supplementary Figs S1–S4; Supplementary Data are available online at www.liebertpub.com/aid).

Phylogenetic analyses of P and S (nt 2848–3215, 1–835) genes revealed that Os/JP/2008, Tk/JP/2009, and Os/JP/2010 were positioned close to HBV/G strains, but Os/JP/2011 and Os/JP/2013 were clustered close to HBV/A strains (Supplementary Data S1 and S2). In contrast, all five were clustered with HBV/A or HBV/G strains when phylogenetic analyses were performed on S (nt 1–835) or C (nt 1814–2491) and X (nt 1374–1835) genes (Supplementary Data S3). Os/JP/2008, Tk/JP/2009, and Os/JP/2010 were positioned close to previously identified Ae/G recombinants and showed similar patterns of recombination, i.e., the recombination points occurred at nt 950–1100 and nt 3100–3200, spanning the 3′ end and the middle section of the S gene, respectively. The crossover patterns of Os/JP/2011 and Os/JP/2013 were distinct from those of Os/JP/2008, Tk/JP/2009, and Os/JP/2010 in that the recombination points lie at nt 1450–1500 and nt 2350–2400 at the 5′ end of the X gene and at the 3′ end of the C gene, respectively (Fig. 2).

Among the five novel recombinants, Os/JP/2008, Tk/JP/2009, and Os/JP/2010 shared >99.9% sequence similarities at the nucleotide level, and showed similar recombination

patterns. In contrast, Os/JP/2011 and Os/JP/2013 shared a high sequence similarity (>99.1%), although their patterns of recombination were different from those of Os/JP/2008, Tk/JP/2009, and Os/JP/2010. The *env* nucleotide sequence similarity of HIV that was coinfected with Os/JP/2008 was 93.8% of that with Os/JP/2010. However, the *env* gene of HIV-1 that was coinfected with Tk/JP/2009 had 81.4% and 82.2% sequence similarities with those with Os/JP/2008 and Os/JP/2010, respectively. Furthermore, the sequence similarity of HIV-1 *env* between Os/JP/2011 and Os/JP/2013 was 83.8%.

Genetic traits of the Ae/G recombinants

The genetic features of HBV/G are as follows: the presence of stop codons at codons 2 (TAA instead of CAA) and 28 (TAG instead of TGG) in the precore region; a 36-bp insertion in the core gene; mutations in the core promoter (A1762T, G1764A); and a 3-bp deletion in the pre-S1 region. All the five Ae/G recombinants identified herein shared the above genetic features. In contrast, the characteristic feature of HBV/Ae, namely a 6-bp insertion at the 3′ end of the core gene, was present only in Os/JP/2011 and Os/JP/2013.

The "Ae genotype regions" (nt 173–926) within Os/JP/2008, Tk/JP/2009, and Os/JP/2010 were genetically similar to that of an isolate identified in the United States in 1998 [accession number JQ707544]. The same genetic region within Os/JP/2011 and Os/JP/2013 was similar to that of an HBV/Ae isolate identified in Japan in 1993 [accession number KC836877]. Interestingly, the nucleotide sequences of HBV/Ae strains that coinfected with Tk/JP/2009, Os/JP/2010, Os/JP/2011, and Os/JP/2013 were highly similar to KC836877 (99.9–100%). We were not able to carry out the analysis on Os/JP/2008 due to a lack of sample.

Coinfection status of the Ae/G recombinant with HBV/Ae and /G

A previous study has shown that HBV A/G recombinants often coinfect with HBV/A and HBV/G.[28] Thus, we next examined whether HBV/A and HBV/G were present in specimens that were positive for Ae/G recombinants. To do this, we developed a PCR system that distinguished genotypes Ae, G, and Ae/G. As expected, all five specimens gave positive results in the Ae/G-specific PCR. Similarly, all five were positive in the Ae-specific PCR. However, the G-specific PCR failed to amplify any of the specimens, although the limit of detection remained to be determined. On the other hand, another PCR designed to amplify the "G genotype region" of Ae/G recombinants was positive for all five specimens that targeted the 36-bp insertion of the core gene. The specificity of PCR was verified by sequencing the amplicons. This suggests that HBV recombinant genotype Ae/G coexisted with Ae, whereas the level of genotype G was below the limit of detection.

To estimate the Ae to Ae/G recombinant ratio, we cloned and sequenced the PCR products amplified using primer set HBVALL9/HBVALL10, which amplifies both genotypes. The number of independent clones tested for Os/JP/2008, Tk/JP/2009, Os/JP/2010, Os/JP/2011, and Os/JP/2013 was 13, 2, 21, 27, and 11, respectively. All the cloned amplicons were derived from Ae/G recombinants, and no amplicons encoding HBV/Ae were found. These data suggest that Ae/G recombinants are circulating dominantly in the infected individuals.

Pathophysiology of Ae/G recombinant HBV infection

We next examined the pathophysiological characteristics of individuals harboring Ae/G recombinants (Table 2). As described above, all five Ae/G recombinant-positive individuals were Japanese MSM who were coinfected with HIV-1. They were aged 24–41 years at the time of serodiagnosis and showed no apparent clinical signs, suggesting that all were in the asymptomatic phase of HIV/HBV coinfection.

Virological analysis revealed that all were infected with HIV-1 subtype B, a common subtype in Europe, the United States, and Japan. The HIV-1 viral load in the plasma ranged from 3.3×10^3 to 7.7×10^4 per ml, which is not very high; however, the HIV-1 infection was not controlled as none of the subjects was taking antiretroviral drugs. The following mutations were identified in the HIV genome: T69N in reverse transcriptase and L10V in the viral protease. These mutations represent common polymorphisms within the HIV-1 genome, although they confer only weak resistance to reverse transcriptase or protease inhibitors.

All specimens were positive for HBsAg and HBcAb, but negative for HBsAb, suggesting that all subjects had an active HBV infection. The HBV viral load in the plasma ranged from 3.59×10^7 to 2.27×10^{10} copies per ml, indicating high levels of HBV replication. No mutations conferring resistance to the nucleoside analogues lamivudine, entecavir, or tenofovir were detected, consistent with the fact that all subjects were treatment naive.

Plasma samples were tested for parameters related to liver function, including AST, ALT, ALP, LDH, and γ-GTP. Most were within the normal range, suggesting that none of the five subjects suffered severe liver dysfunction at the time of diagnosis, although mildly increased AST or AST, ALP, and γ-GTP levels were observed in the subjects infected with Os/JP/2013 or Tk/JP/2009.

Discussion

HIV-1 was shown to spread in a specific community with high-risk behaviors, including MSM. Such individuals have high probabilities of being coinfected with HIV-1 and HBV based on the similarity of their infection route. In fact, the seroprevalence of HBV among HIV-positive individuals was 63.2%.[26] The coinfection risk of multiple HBV genotypes is also high. In Canada, 67% of HBV/G-positive individuals were male homosexuals and were coinfected with an HBV/A or A/G recombinant.[28] In Japan, the coinfection of HBV/G and /A has also been reported.[24] In individuals coinfected with distinct HBV genotypes, a recombinant HBV may be generated. HBV recombinants originating from HBV genotypes A and D or B and C have been already reported. An intergenotype HBV recombinant made from genotypes A, C, and G is now categorized as a distinct genotype I. The crossover patterns of recombinant viruses vary[1]; however, some regions of the HBV genome appear to favor intergenotype or intragenotype recombination.

In line with previous reports, the crossover points of the five novel Ae/G recombinants described herein were positioned at or close to genetic regions in which recombinations occur frequently.[42] According to the recombination patterns, it seems likely that Os/JP/2008, Tk/JP/2009, and Os/JP/2010 originated from the same ancestor and the ancestor of Os/JP/2011 and Os/JP/2013 was different from that of the other

TABLE 2. LABORATORY FINDINGS OF INDIVIDUALS INFECTED WITH NOVEL AE/G RECOMBINANTS

Patient	*1*	*2*	*3*	*4*	*5*
Virus nomenclature	Os/JP/2008	Tk/JP/2009	Os/JP/2010	Os/JP/2011	Os/JP/2013
Geographic origin	Osaka	Tokyo	Osaka	Osaka	Osaka
Gender	M	M	M	M	M
Age	39	32	41	34	24
Risk factor	MSM	MSM	MSM	MSM	MSM
HBV					
Serology					
HBsAg	+	+	+	+	+
HBcAb	+	+	+	+	+
HBsAb	–	–	–	–	–
Viral load (copies/ml)	2.3×10^{10}	3.6×10^{7}	ND	7.2×10^{9}	9.0×10^{9}
Drug resistance, 3TC,ETV,TFV	–	–	–	–	–
Coinfection with	+	+	+	+	+
HIV					
Subtype	B	B	B	B	B
Viral load (copies/ml)	4.1×10^{4}	4.2×10^{3}	ND	7.7×10^{4}	3.3×10^{3}
Drug resistance					
RT	–	–	–	T69N	V179A
Protease	L10V,L63A	L63T,V77I,I93L	L10V,L63A	K43R,L63A,I64V	L63T,I93L
TPAb[a]	–	+	–	–	+
Risk factor	MSM	MSM	MSM	MSM	MSM
Biochemical data					
AST(10 ~ 40)[b] (IU)	20	60	ND	40	66
ALT(5 ~ 45)[b] (IU)	20	20	ND	20	34
ALP(104 ~ 338)[b] (IU)	200	340	ND	200	228
LDH(120 ~ 240)[b] (IU)	140	60	ND	80	124
γ-GTP(10 ~ 40)[b] (IU)	40	120	ND	20	26

[a]Anti-*Treponema pallidum* antibody.
[b]IU, international unit. Normal range is shown.
M, man; 3TC, lamivudine; ETV, entecavir; TFV, tenofovir; RT, reverse transcriptase; MSM, men who have sex with men; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; γ-GTP, gamma-glutamyl transferase; ND, no date.

three. Considering the high-risk behavior and the molecular epidemiological data, it was suggested that HIV-1 and HBV were not always cotransmitted in the MSM populations. We described five novel HBV Ae/G recombinants in Japanese MSM coinfected with HIV-1, and the recombination patterns of these recombinants were totally distinct from five HBV Ae/G recombinants identified previously. These data suggest that novel recombinants should have been generated independently in distinct MSM communities. It was noted that HBV genotyping using only a part of the viral genome may be misleading.[43]

HBV/G, which often infects homosexual individuals, is also present in individuals harboring HBV/A.[6,30,34,44,45] This is partly due to the poor replicative capacity of HBV/G. The presence of HBV/A significantly increases the rate of HBV/G replication in hepatocytes, although infection with HBV/G alone is possible.[46–49] Genetic rearrangements between HBV/A and HBV/G can occur during persistent coinfection. Therefore, we examined how novel Ae/G recombinants were generated and transmitted in the Japanese MSM population. To do this, we focused our analysis on genetic regions derived from HBV/Ae, but not from HBV/G, since the genetic diversity of the HBV/G genogroup is poor. Molecular epidemiological data suggested that Os/JP/2008, Tk/JP/2009, and Os/JP/2010 evolved from an ancestral Ae/G recombinant in North America that was then introduced into the Japanese population. In contrast, the ancestor of Os/JP/2011 and Os/JP/2013 viruses may be generated independently in the domestic MSM population, which was then spread along with the parental domestic strain of HBV/Ae.

The PCR experiments showed that HBV Ae/G recombinants were present dominantly over the HBV/Ae, and the levels of HBV/G were under the limit of detection in all five cases, although these data had some limitations involving the number of examined amplicons as well as intrinsic problems in PCR. However, the dominance of the Ae/G recombinant was probably due to its replicative capacity and/or selective advantage *in vivo*. It was likely that host immunity provided a primary selection pressure. A similar phenomenon was reported previously wherein a selection pressure may be partly attributed to antiviral agents.[36] The finding that HBV/Ae was present, albeit at a much lower frequency, suggests that Ae/G recombinants still require HBV/Ae for efficient replication, as does HBV/G.

Novel HBV recombinants were found in the specimens collected for the anonymous HIV-1 testing. Thus, we have limited clinical information about the examinees. Knowing these limitations, biochemical findings indicated that coinfection of the HBV Ae/G recombinant and HIV-1 did not always result in a rapid progress of disease in a short period of time. Some HBV genotypes show a severe clinical course.[50] Here, we were unable to determine whether these novel Ae/G HBV recombinants are more pathogenic than other HBV genotypes. This is primarily because the specimens were

collected during the asymptomatic phase of HIV/HBV infection and the clinical course of the infected individuals was not monitored after virus detection. This will be addressed in a future study.

A previous report from Brazil identified HBV/G harboring mutations conferring lamivudine resistance (L180M and M204V). It is notable that two of the three reported cases were HIV positive.[34] It is a concern that the potential spread of novel Ae/G recombinants among Japanese HIV-positive MSM may result in them acquiring drug-resistant mutations that limit treatment options; however, we found no major drug-resistant mutations in any of the five novel Ae/G recombinants identified herein. Taken together, the results of the present study suggest that the molecular epidemiology of HBV should be studied in more detail, especially in HIV-1-positive individuals.

Sequence Data

GenBank accession numbers for the full genome sequences obtained in this study are as follows: AB933279–AB933283. GenBank accession numbers for the Ae sequences obtained in this study are LC029424–LC029427.

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Author Disclosure Statement

No competing financial interests exist.

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