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Short Communication

Identification of novel recombinants of hepatitis B virus genotypes F and G in human immunodeficiency virus-positive patients from Argentina and Brazil

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Hepatitis B virus (HBV) genotype G (HBV/G) infection is almost always detected along with a co-infecting HBV strain that can supply HBeAg, typically HBV/A2. In this study we describe, in two human immunodeficiency virus (HIV)-positive patients from Argentina and Brazil, the first report of HBV/G infection in Argentina and co-circulation of HBV/G, HBV/F and G/F recombinants in the American continent. HBV isolates carrying the 36 bp insertion of HBV/G were the most prevalent in both patients, with >99 % of colonies hybridizing to a probe specific for this insertion. Phylogenetic analyses of full-length genomes and precore/core fragments revealed that F4 and F1b were the co-infecting subgenotypes in the Brazilian and Argentinian patients, respectively. Bootscanning analysis provided evidence of recombination in several clones from both patients, with recombination breakpoints located mainly at the precore/core region. These data should encourage further investigations on the clinical implications of HBV/G recombinants in HBV/HIV co-infected patients.

Hepatitis B virus (HBV) infection is a major cause of chronic liver disease, including cirrhosis and hepatocellular carcinoma, affecting >350 million people (WHO, 2008). Eight genotypes (HBV/A to H) have been identified, representing divergence of >8 % in complete genomes (Arauz-Ruiz *et al.*, 2002; Norder *et al.*, 1994; Stuyver *et al.*, 2000). Two additional genotypes, I and J, have been proposed (Olinger *et al.*, 2008; Tatematsu *et al.*, 2009; Huy *et al.*, 2008). There is diversity within genotypes, some genotypes being divided into subgenotypes (Norder *et al.*, 2004). Genotypes/subgenotypes have distinct geographical distributions: HBV/A and HBV/D are found worldwide, HBV/B and HBV/C are found in east/south-east Asia, HBV/E in west/central Africa, and HBV/F and HBV/H in native populations of the Americas (Araujo *et al.*, 2011; Kramvis *et al.*, 2005). HBV/G was described by Stuyver *et al.* (2000) and, despite low prevalence, seems ubiquitous, as it has been reported in Europe (Lacombe *et al.*, 2006; Vieth *et al.*, 2002), the Americas (Alvarado-Esquivel *et al.*, 2006; Alvarado Mora *et al.*, 2011; Bottecchia *et al.*, 2008; Chu *et al.*, 2003; Osiowy *et al.*, 2008), Asia (Shibayama *et al.*, 2005; Toan *et al.*, 2006) and Africa (Olinger *et al.*, 2006; Toan *et al.*, 2006). HBV/G is frequently reported in patients infected with human immunodeficiency virus (HIV) (Dao *et al.*, 2011; Zehender *et al.*, 2003), particularly in men who have sex with men (MSM) (Bottecchia *et al.*, 2008; Osiowy *et al.*, 2008). Patients co-infected with HBV/G and HIV have an increased risk of liver fibrosis compared with those infected with other HBV genotypes

The GenBank/EMBL/DDBJ accession numbers for the HBV sequences obtained in this study are HE981171–HE981189.

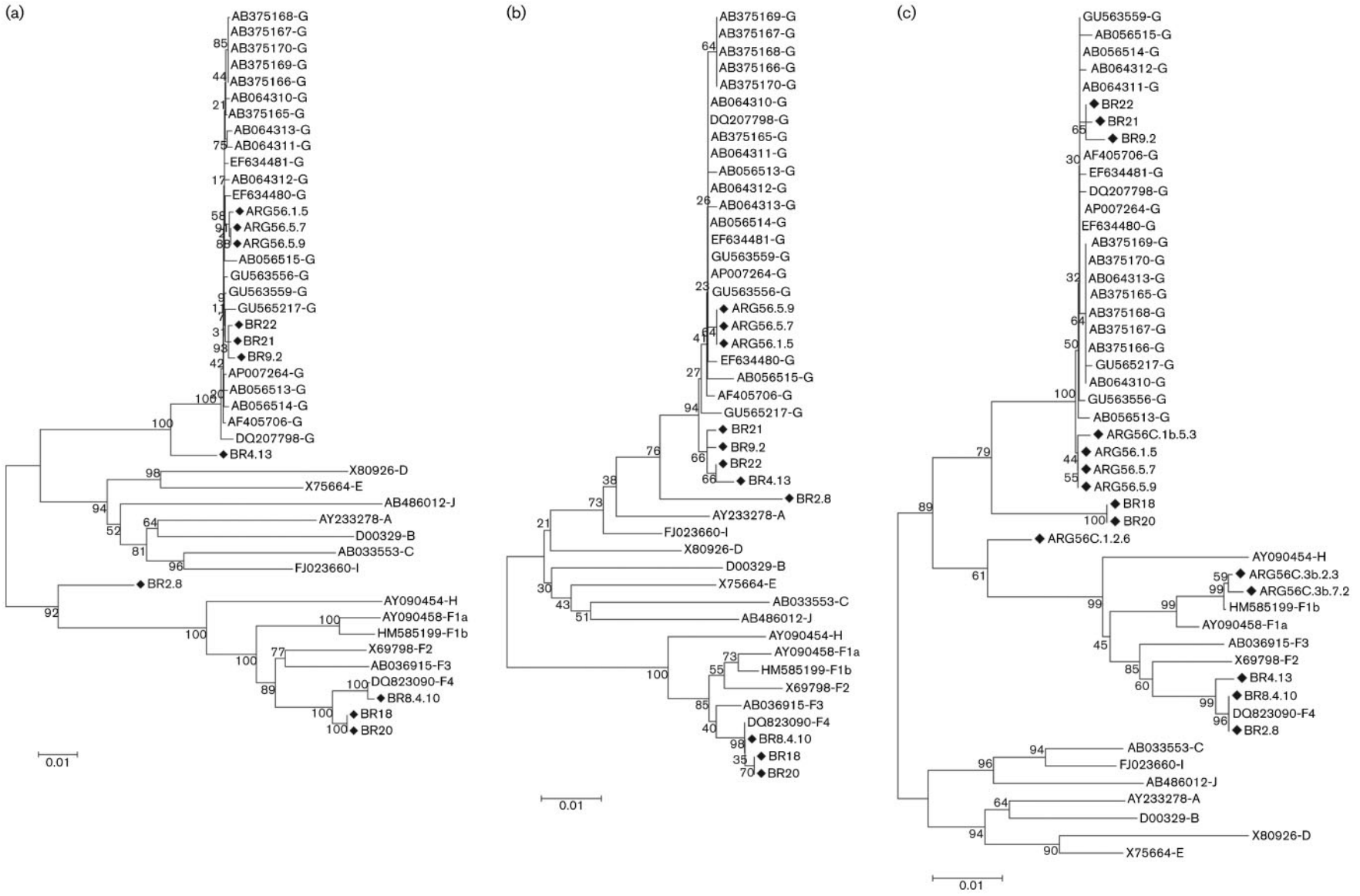
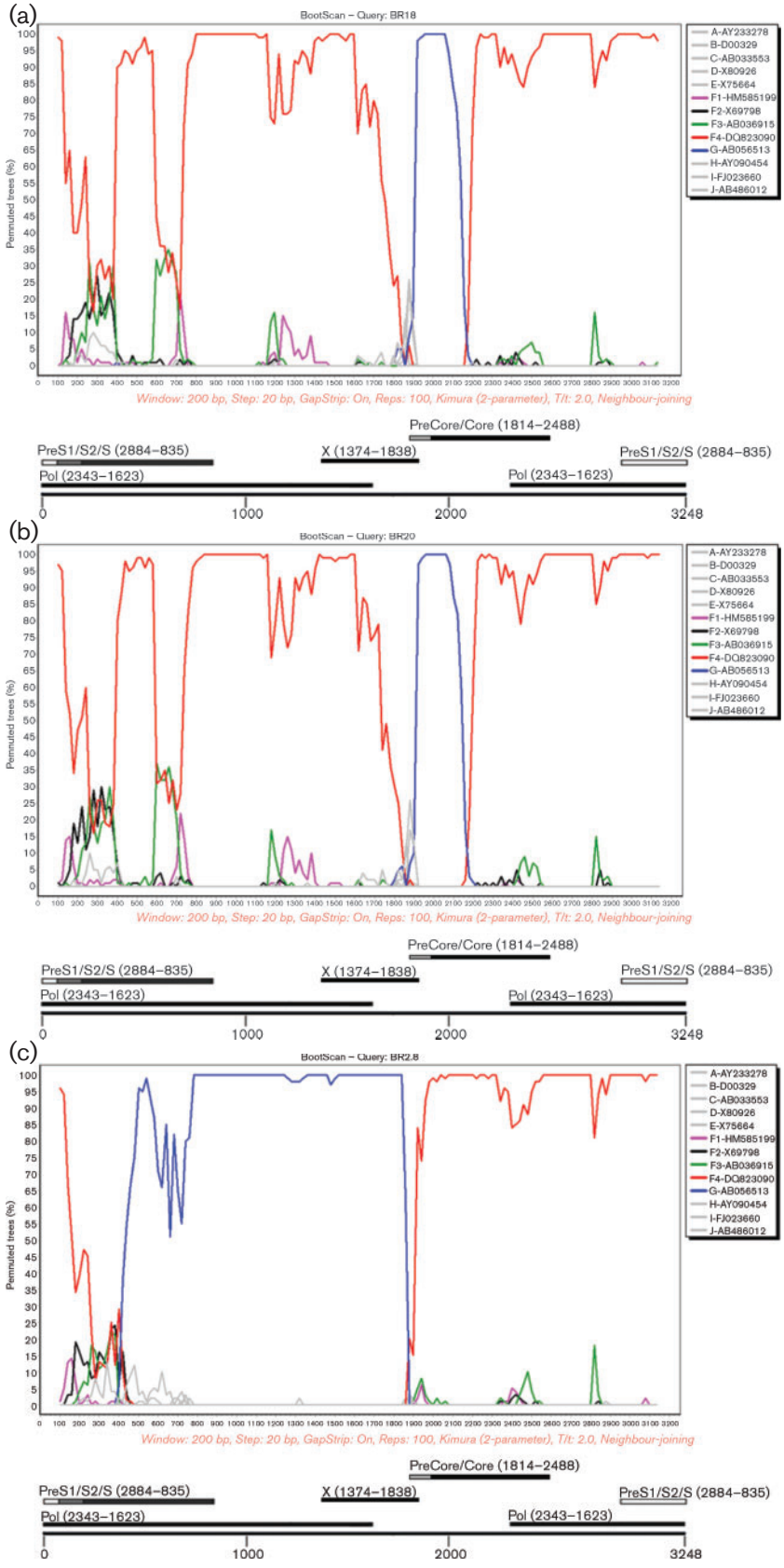


Fig. 1. Phylogenetic analysis of HBV sequences using the neighbour-joining method. GenBank accession numbers for the prototype strains representing genotypes A–J are provided in the figure; ◆ identifies sequences generated in this study. HBV sequences from Brazil and Argentina are denoted BR and ARG, respectively, followed by the clone number. Values at internal nodes indicate percentages of 1000 bootstrap replicates that support the branch. Bars, number of nucleotide substitutions per site. (a) Analysis of the full-length sequences. (b) Analysis of the complete S (surface) gene region. (c) Analysis of the complete C (core) gene region.


(a)
BootScan – Query: BR18
Permuted trees (%)
A-AY233278
B-D00329
C-AB033553
D-X80926
E-X75664
F1-HM585199
F2-X69798
F3-AB036915
F4-DQ823090
G-AB056513
H-AY090454
I-FJ023660
J-AB486012
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbour-joining
PreCore/Core (1814–2488)
PreS1/S2/S (2884–835)
X (1374–1838)
PreS1/S2/S (2884–835)
Pol (2343–1623)
Pol (2343–1623)
0
1000
2000
3248
(b)
BootScan – Query: BR20
Permuted trees (%)
A-AY233278
B-D00329
C-AB033553
D-X80926
E-X75664
F1-HM585199
F2-X69798
F3-AB036915
F4-DQ823090
G-AB056513
H-AY090454
I-FJ023660
J-AB486012
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbour-joining
PreCore/Core (1814–2488)
PreS1/S2/S (2884–835)
X (1374–1838)
PreS1/S2/S (2884–835)
Pol (2343–1623)
Pol (2343–1623)
0
1000
2000
3248
(c)
BootScan – Query: BR2.8
Permuted trees (%)
A-AY233278
B-D00329
C-AB033553
D-X80926
E-X75664
F1-HM585199
F2-X69798
F3-AB036915
F4-DQ823090
G-AB056513
H-AY090454
I-FJ023660
J-AB486012
Window: 200 bp, Step: 20 bp, GapStrip: On, Reps: 100, Kimura (2-parameter), T/t: 2.0, Neighbour-joining
PreCore/Core (1814–2488)
PreS1/S2/S (2884–835)
X (1374–1838)
PreS1/S2/S (2884–835)
Pol (2343–1623)
Pol (2343–1623)
0
1000
2000
3248

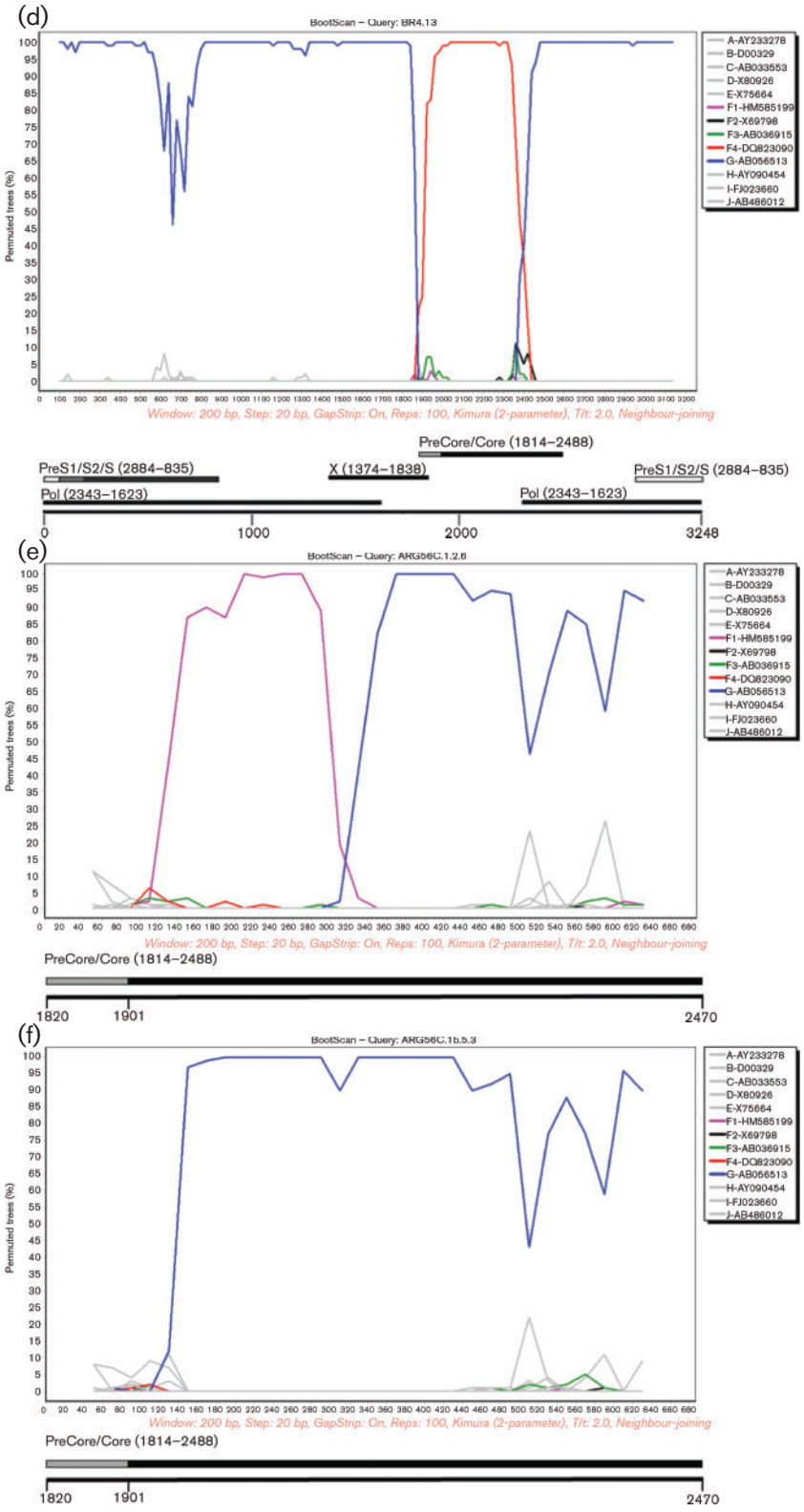


Fig. 2. Bootscan plots for the Brazilian (a) BR18, (b) BR20, (c) BR2.8, (d) BR4.13 and Argentinian (e) ARG56C.1.2.6 and (f) ARG56C.1b.5.3 clones. Each genotype (A–J) was represented by prototype sequences indicated in the legend. The parameters used for the analysis are shown at the bottom of the figures. A window size of 200 bp was chosen. No difference was observed in breakpoint profiles when a window size of 400 bp was applied. Linear physical maps of HBV are provided and ORFs that encode more than one protein are indicated in different colours. Numbering starts from the (often hypothetical) *Eco*RI restriction site.

(Dao *et al.*, 2011; Lacombe *et al.*, 2006). HBV/G is unusual in several aspects: it has a 36 bp insertion at the beginning of the core gene and two stop codons in the precore region (codons 2 and 28), preventing expression of HBeAg (Kato *et al.*, 2002). Moreover, it almost exclusively co-infects with another HBV genotype, usually HBV/A (Kato *et al.*, 2002; Osiowy *et al.*, 2008), but HBV/C (Suwannakarn *et al.*, 2005), HBV/F (Fallot *et al.*, 2012) and HBV/H (Sánchez *et al.*,

2007) have also been reported. Recombination between HBV/G and the co-infecting genotype has been observed, especially in patients with HIV (Fallot *et al.*, 2012; Martin *et al.*, 2011; Osiowy *et al.*, 2008). Recombination is an important element of HBV genetic variability and may have clinical implications (Fallot *et al.*, 2012; Kay & Zoulim, 2007; Simmonds & Midgley, 2005). Herein, we describe the first report of HBV/G infection in Argentina and co-circulation of HBV/G, HBV/F and G/F recombinants in the American continent, providing full-length genomic sequences of several HBV recombinant and non-recombinant isolates.

HBV isolates were obtained from two patients respectively referred to the Medical Diagnostic Center of High Complexity, Rosario, Argentina, and Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil. Both patients were male, HIV-positive and seropositive for HBsAg, HBeAg and anti-HBc, with serum HBV loads $>10^8$ copies ml^{-1}. The Brazilian patient is an MSM; the infection route for the Argentinian patient was not available. The protocol was approved by Local Ethics Committees and informed consents were obtained from the patients. HBV DNA was extracted from 0.2 ml serum using a High Pure Viral Nucleic Acid kit (Roche Diagnostics). The full-length HBV genome was amplified as described previously (Günther *et al.*, 1995), with or without prior rolling-circle amplification (RCA) (Margeridon *et al.*, 2008). The precore/core region (positions 1824–2467) of the Argentinian patient was amplified by subgenomic PCR. The DNA polymerase used was Herculase II fusion DNA polymerase (Agilent) for 40 cycles. Products were cloned into pUC19 (Promega) or pSC-B (Agilent). Clones were screened by colony hybridization using a radioactive probe specific for the 36 bp insertion of HBV/G, stripped, then rehybridized with another probe that detects all HBV genotypes. Clones of interest were sequenced (GenBank accession numbers HE981171–HE981189). Phylogenetic/molecular evolutionary analyses used MEGA version 4.1 (Tamura *et al.*, 2007). Phylogenetic trees were obtained using the neighbour-joining method (1000 bootstrap replicates) and mean genetic distances were estimated by Kimura two-parameter analysis. Bootscan software (SimPlot v. 3.5.1) was used to identify intergenotypic recombination (Lole *et al.*, 1999). This method compared a putative recombinant sequence with ten reference sequences corresponding to HBV/A–HBV/J.

For the Brazilian patient, full-length HBV genomes amplified by PCR were cloned. Twenty-eight HBV DNA-positive colonies were initially isolated and RFLP analysis (*Nde*I and *Stu*I) suggested that all but two clones were 'pure' HBV/G. Four clones (BR18 and 20–22) were selected for sequencing. Phylogenetic analysis confirmed that clones BR21 and BR22 are HBV/G (Fig. 1a–c), whereas clones BR18 and BR20 show discordant results with the S- and C-genes (Fig. 1b, c). For the C-gene, BR18 and BR20 cluster with HBV/G strains, but in the S-gene they cluster with HBV/F4 sequences, indicating that both are recombinant strains and that the co-infecting strain is HBV/F4. To find a full-length HBV/F4 clone, as many clones as possible were screened using hybridization with an HBV/G-specific probe and an HBV/F4-specific probe. Fifty-five colonies hybridized with the genotype G probe and only one colony (BR8.4.10) hybridized with the HBV/F4 probe. Sequencing showed that this clone was 'pure' HBV/F4, suggesting that HBV/F4 strains represent about 2 % of HBV genomes in this patient.

For the Argentinian patient, direct sequencing showed that the patient was infected with HBV/G. Twenty-three full-length HBV-DNA positive colonies were isolated and hybridization suggested that all were HBV/G, confirmed by sequencing three clones (ARG56.1.5, ARG56.5.7 and ARG56.5.9; Fig. 1a–c). To find the co-infecting genotype in this patient and to increase the numbers of informative colonies, subgenomic C-gene PCR products were cloned. Of >400 HBV-positive colonies, only four hybridized with the pan-genomic probe and not with the HBV/G probe. These clones were sequenced and phylogenetic analysis indicated that two (ARG56C.3b.2.3 and ARG56C3b.7.2) are 'pure' HBV/F1b, whilst two (ARG56C.1.2.6 and ARG56C.1b.5.3) are HBVG/F1b recombinants with different breakpoints (Figs 1c and 2e, f).

To enrich for full-length non-G strains, especially HBV/F1b strains in the Argentinian patient, serum extracts were first subjected to a modified RCA (Margeridon *et al.*, 2008; N. Martel, C. Trepo & A. Kay, unpublished data). RCA products were cut with *Nde*I, which has a site in the 36 bp insert specific to HBV/G but is absent in HBV/F, before being amplified by genomic PCR. By these means, two new G/F4 recombinants (BR2.8 and BR4.13) were found. An HBV/G strain (BR9.2) was also isolated and sequenced, and was found to contain a mutation affecting the *Nde*I site. Several non-G clones were isolated from the Argentinian patient and four were sequenced (ARG56K4.3, ARG56S2.3, ARG56S3.8 and ARG56S5.9). All are 'pure' HBV/F1b. Despite many efforts, no full-length G/F1b recombinants could be isolated.

Bootscanning was performed to locate breakpoints of genomic recombination more accurately (Fig. 2a–f). Clones BR18 and BR20 have an HBV/G sequence from position 1845 to 2132 and are HBV/F4 in origin in the rest of the genome (Fig. 2a, b). Inversely, clone BR4.13 is HBV/F4 from position 1817 to 2442 (Fig. 2d). Clone BR2.8 has an HBV/G sequence from position 493 to 1816 and it is the only clone with a recombination breakpoint in the S-gene (Fig. 2c). Clone BR8.4.10 is HBV/F4 and clones BR21, BR22 and BR9.2 are HBV/G, with no signs of recombination. Concerning the precore/core subgenomic clones from Argentina (positions 1824–2467), clone ARG56C.1.2.6 is HBV/F1b from position 1824 to position 2154 and HBV/G thereafter (Fig. 2e). Clone ARG56C.1b.5.3 is clearly HBV/G from position 1970 to the end of the fragment. However, at the 5′ end it does not have the 36 bp insertion specific to HBV/G and has a wild-type codon at codon 28 of precore region (Fig. 2f). This region is well-conserved in all HBV genotypes and, whilst it is obviously a recombinant, we cannot conclude that it is

F1b/G. Clones ARG56C.3b.2.3 and ARG56C.3b.7.2 are HBV/F1b throughout the amplified fragment.

Due to the geographical proximity of Brazil and Argentina, we investigated whether Brazilian and Argentinian HBV/G isolates are genetically closer than HBV/G isolates from other regions of the world. Phylogenetic analysis with HBV/G full-length genomes from our study and database sequences from different geographical regions showed that Brazilian and Argentinian HBV/G isolates were not related particularly closely (Fig. 1a), suggesting that HBV/G may have had different introduction routes into the continent. This is corroborated by Alvarado Mora *et al.* (2011), who showed that different lineages of HBV/G circulate in Colombia. Moreover, the mean genetic distance between Brazilian and Argentinian full-length HBV/G clones (excluding recombinants) and 21 HBV/G sequences from other regions throughout the world was studied (Table 1). Similar values were observed between all geographical groups analysed (Argentina, Brazil, Europe, Japan, Mexico and the USA), varying from 0.002 ± 0.001 to 0.004 ± 0.001 (Table 1). This is not unexpected, as HBV/G has been described as being genetically homologous among worldwide isolates (Lindh, 2005).

Important mutations were observed in several clones. The Brazilian HBV/G clones (BR21, BR22 and BR9.2) as well as the G/F4 recombinant BR4.13 all have a 12 nt deletion from position 54 to 65 in the pre-S2 region. This leads to deletion of pre-S2 residues 19–22 and change of residue 18 from Arg to Ser. These clones, as well as the G/F4 recombinant BR2.8, also have a missense mutation in the polymerase gene leading to rtL180M, but do not possess the lamivudine-resistance mutation rtM204V/I. In addition, clones BR22 and BR4.13 have a stop codon in HBsAg at codon 182. The Argentinian HBV/G clones do not exhibit any obviously significant mutations. However, for the HBV/F1b clones, only ARG56S3.8 is 'wild type' throughout the genome, as are clones ARG56C.3b.2.3 and ARG56C.3b.7.2 in the subgenomic fragment sequenced. For the other full-length HBV/F1b clones, ARG56S5.9 possesses a 12 bp deletion (positions 2167–2178) in the C gene, and ARG56K4.3 and ARG56S2.3 have two identical 1 bp insertions in the C gene (between positions 1921 and 1922, and 2189 and 2190) and also have a stop codon at codon 2 of pre-S2. It should be noted that all mutations in the C gene are not overlapped by the Pol gene. Consistent with HBV/F1b genomes being rare in Argentinian patients and that there are major perturbations in their C-gene, affecting HBeAg expression, HBeAg levels are very low. On the other hand, the Brazilian patient has a higher prevalence of HBV/F4 and no obvious defects in the C-gene, and HBeAg levels are higher.

This is the first report of HBV/G infection in Argentina. In South America, HBV/G infection has been reported only in some patients from Brazil (Bottecchia *et al.*, 2008; Silva *et al.*, 2010) and Colombia (Alvarado Mora *et al.*, 2011).

Initiation, but not maintenance, of chronic HBV infections seems to require expression of HBeAg (Hadziyannis, 2011). As HBV/G strains are constitutively unable to express HBeAg, in chronic infections they are invariably found to be associated with another co-infecting HBV genotype capable of furnishing HBeAg *in trans*. In the vast majority of HBV/G chronic infections, this co-infecting strain is subgenotype A2 (Kato *et al.*, 2004, 2002; Martin *et al.*, 2011; Osiowy *et al.*, 2008). It was therefore unexpected that, in two cases described here from two different South American countries, the co-infecting strain was HBV/F, although co-infection with HBV/G and either HBV/A2 or HBV/H has been reported in Mexico (Sánchez *et al.*, 2007). The obvious explanation is that HBV/G can associate with prevalent local strains capable of supplying HBeAg. This could be the case in Argentina, where HBV/F (mainly HBV/F1b and HBV/F4) is the most prevalent HBV genotype among Argentinian chronic hepatitis B patients (Mbayed *et al.*, 2001; Telenta *et al.*, 1997) and blood donors (França *et al.*, 2004). However, HBV/G infections are associated with HBV/A2 in Japan (Kato *et al.*, 2002) where this subgenotype is rare, and in Brazil HBV/G is extremely rare and HBV/F prevalence is low (<15 %) countrywide (Araujo *et al.*, 2004; Mello *et al.*, 2007; Paraná & Almeida,

Table 1. Mean genetic distances between groups of full-length HBV genotype G sequences from different geographical regions

Mean genetic distances were estimated by Kimura two-parameter analysis. Sequences included in the analysis: Argentina, clones ARG56.1.5, ARG56.5.7, ARG56.5.9; Brazil, clones BR21, BR22, BR9.2; Europe, GenBank accessions AF405706, DQ207798, GU563559, GU563556, EF634481, EF634480, GU565217; Japan, AP007264; Mexico, AB375170, AB375169, AB375168, AB375167, AB375166, AB375165; USA, AB064313, AB064312, AB064311, AB064310, AB056515, AB056514, AB056513; World, all sequences indicated above.

	Argentina	Brazil	Europe	Japan	Mexico	USA	World
Argentina	0.000						
Brazil	0.004	0.000					
Europe	0.004	0.004	0.000				
Japan	0.003	0.003	0.002	0.000			
Mexico	0.003	0.003	0.003	0.002	0.000		
USA	0.003	0.004	0.003	0.002	0.002	0.000	
World	0.003	0.004	0.003	0.002	0.003	0.003	0.000

2005). In addition, the related HBV/F subgenotype in the Brazilian patient was F4 and not F2, which has been found to be most prevalent in Brazil (Mello *et al.*, 2007).

It is of note that, in both the Argentinian and the Brazilian patients, the HBV/F strains form minor populations (1–2 % of HBV genomes). For the Argentinian patient, this can be explained by mutations in the core gene found in three of the four full-length genomes. These isolates can only persist if HBcAg is supplied *in trans*, which is possible given that genotype G strains overexpress HBcAg (Kremsdorf *et al.*, 1996), but means that the defective HBV/F1b strains need to co-infect hepatocytes along with a HBV/G strain. For the Brazilian patient, the answer lies perhaps with the rtL180M mutation found in all HBV/G clones. This is a compensatory mutation associated with lamivudine resistance, restoring replication fitness to HBV polymerase harbouring rtM204V/I (Ono *et al.*, 2001). In this study, the authors also showed that complete genomes carrying rtL180M without rtM204V/I produced higher levels of HBV-DNA than wild-type genomes. Therefore, the presence of rtL180M alone in the Brazilian HBV/G strain may have led to a selective advantage over co-infecting HBV/F strains.

It is also noteworthy that novel intergenotypic G/F recombinants in both patients were identified. Are these real recombinants or have they been generated by strand switching during PCR amplification? This cannot be excluded for the Argentinian patient, as recombinants were identified only by subgenomic PCR. However, for the Brazilian patient there are several arguments against this possibility. First, to obtain the recombinants described, two strand switches would have to occur in the same PCR. Secondly, the HBV/F genomes are very rare and strand switching from HBV/G to HBV/F is highly unlikely. This argument also applies to the Argentinian patient. Thirdly, and perhaps most importantly, 'pure' HBV/F genomes should be present in greater quantities than artefactual recombinant genomes, and this is not the case.

These are the first HBV G/F recombinant genomes characterized from the Americas. Recombination between HBV/G and HBV/F has previously been reported only once, in an HIV-positive patient, surprisingly from France (Fallot *et al.*, 2012). Dual HBV infection and genomic recombination between different genotypes have been increasingly documented (Simmonds & Midgley, 2005; Sugauchi *et al.*, 2003; Yang *et al.*, 2007). Recombination has been found to occur more frequently in certain 'hot-spot' regions of the viral genome, such as the core region (Bowyer & Sim, 2000; Garmiri *et al.*, 2009; Luo *et al.*, 2004), pre-S1 (Chen *et al.*, 2004), pre-S2/S (Chen *et al.*, 2006; Wang *et al.*, 2005), polymerase (Kurbanov *et al.*, 2005; Magiorkinis *et al.*, 2005) and X (Martin *et al.*, 2011), and has been considered a significant source of HBV genetic variability (Simmonds & Midgley, 2005). In our study, all but one G/F recombinant clones had recombination breakpoints in the core region (Fig. 2a–f).

Dual HBV infection has been reported frequently in HBV/HIV co-infected individuals (Fallot *et al.*, 2012; Martin *et al.*, 2011), and lowered immunity and multiple partners may render these patients more susceptible to mixed infections. An association between HBV genotypes and clinical outcomes, activity of liver disease, HBV replication and treatment responses has been demonstrated (Lin & Kao, 2011; Mayerat *et al.*, 1999; McMahon, 2009). It has also been proposed that modes of transmission may influence HBV virulence by favouring some genotypes over others (Araujo *et al.*, 2011). In particular, a correlation between HBV genotype and sexual transmission has been observed for HBV/G, which is highly associated with MSM as a risk factor for infection (Bottecchia *et al.*, 2008; Osiowy *et al.*, 2008; Sánchez *et al.*, 2007; Shibayama *et al.*, 2005). Also, HBV/G infection has been correlated with more advanced fibrosis of the liver in HIV co-infected patients (Dao *et al.*, 2011; Lacombe *et al.*, 2006). Here, the Brazilian patient was an MSM with elevated ALT (alanine transaminase) levels, but without liver fibrosis. These data for the Argentinian patient were not available.

Hybrid HBV strains resulting from genomic recombination between different genotypes may enhance their virulence, e.g. by harbouring mutations that lead to antiviral resistance or increased replication capacity of the recombinant genomes. The effects of recombination events found in HBV/G infection are currently unknown, and the implications of these recombinants on pathogenesis and disease progression, especially in HBV/HIV co-infected patients, warrant further study.

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