SUPPLEMENTARY DATA FILE

Illumina and Nanopore methods for whole genome sequencing of hepatitis B virus (HBV)

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Suppl Methods 1: Sanger sequencing

We used a pan-genotypic Sanger sequencing approach adapted from Chook *et al* [1] (Suppl Table 2), in which the HBV genome is sequenced as seven overlapping fragments by nested PCR. We added an additional fragment (derived using primer sets E and 7) to the original protocol to improve coverage of the long region amplified using primers 595f and 1797r. Primer sites are shown in Suppl Fig 3 and PCR reactions were performed as previously described [1].

We stained amplified products to verify by agarose gel electrophoresis under UV light, and sequenced using an ABI 3730 / 3730xl capillary sequencer (Department of Zoology, University of Oxford). We aligned fragments using SSE software [2] and derived a consensus sequence. Errors in Sanger sequences (defined as differences from Illumina-derived consensus) largely corresponded to sites of sequencing primers (Suppl Fig 4).

Suppl Methods 2: HBV-specific qPCR

We used HBV-specific qPCR to measure the yield of HBV DNA after rolling circle amplification (RCA) and compared with the non-amplified extracted DNA sample. The qPCR was adapted from Garson *et al*. [3], using a SYBR Green approach to target a 98 bp region of the HBV surface (S) gene. We set up reactions using 2 µl sample DNA and 10 µl Power SYBR Green (Thermo Fisher) and performed thermal cycling using a LightCycler® 480 System, with the following conditions: 1 cycle at 95°C for 10 minutes, and 45 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Prior to qPCR, RCA samples were diluted 100-fold to reduce the background DNA levels for SYBR Green qPCR, which were then compared with non-amplified extracted DNA from the same sample.

Suppl Table 1: Relationship between number of genomes in HBV concatemer, number of Nanopore reads with at least that number of genomes, and average error rate of the Nanopore reads after correction. Data based on sample from an adult with chronic HBV infection with viral load >10^8 IU/ml, recruited in Oxford (sample ID 1331). To correct the reads, the consensus within each concatemer has been used.

Number of complete genomes (g) in concatemer	**3**	**4**	**6**	**8**
Number of reads with ≥ g complete genomes	208	158	84	41
Average error rate after consensus correction (%)	0.88	0.73	0.57	0.51

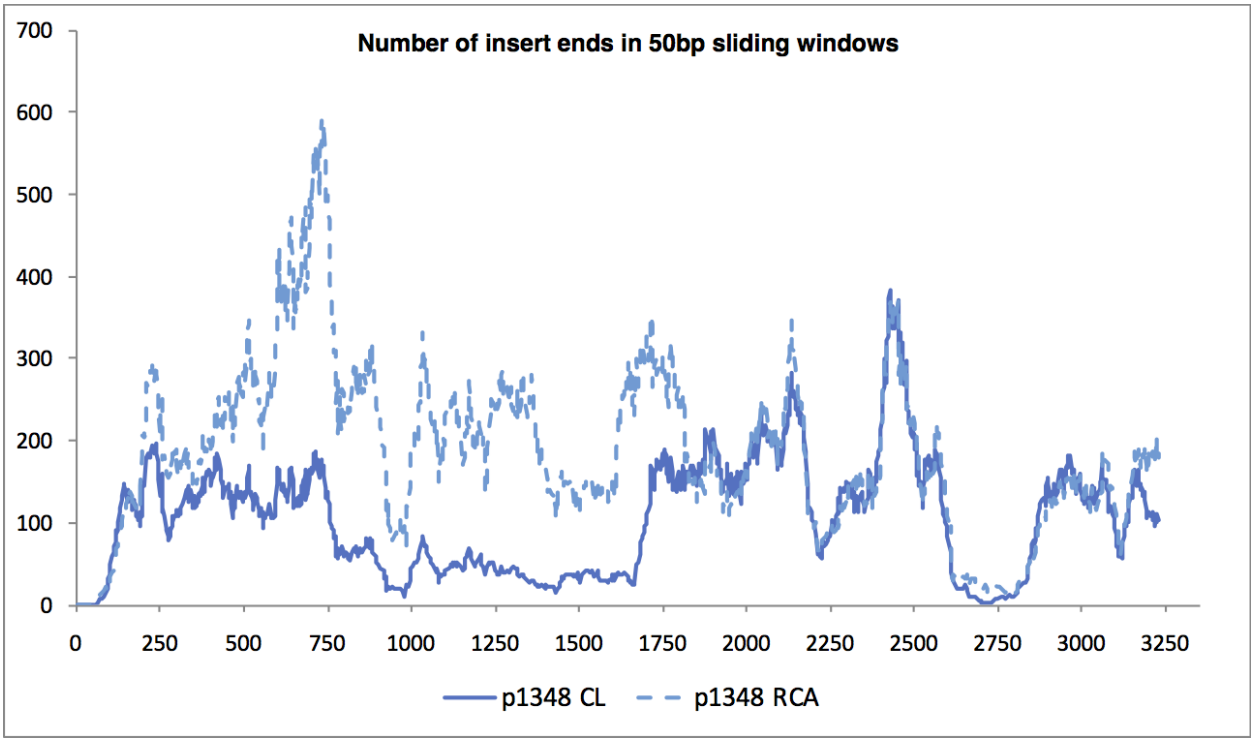
Suppl Table 2: Primers used for Sanger sequencing of full length HBV genome. Primers adapted from Chook *et al*.[1] The primers used for sequencing each amplicon are indicated (*). Primer 1797 (**) is used twice in the first round reactions, as a part of set A and set E.

	Outer primers			**Inner primers**			
Set	**Primer**	**Sequence**	**Tm (°C)**	**Set**	**Primer**	**Sequence**	**Tm (°C)**
A	251F	GACTYGTGGTGGACTTCTC	54	**1**	251F*	GACTYGTGGTGGACTTCTC	54
					1190R	TCAGCAAAYACTYGGCA	
	1797R**	CCAATTTMTGCYTACAGCCTC		**2**	595F*	CACHTGTATTCCCATCCCA	54
					1797R	CCAATTTMTGCYTACAGCCTC	
B	2300F	CCACMWAATGCCCCTATC	50	**3**	2807F*	CGCHTCATTTTGYGGGTCAC	52
					617R	GAYGAYGGGATGGGAATACA	
	654R	GSCCCAMBCCCATAGG		**4**	2300F*	CCACMWAATGCCCCTATC	50
					215R	AGRAAMACMCCGCCTGT	
C	1859F	ACTNTTCAAGCCTCCRAGCTG	52	**5**	1877F	CTGTGCCTTGGRTGGCTT	52
	2835R	GTTCCCAVGWATAWGGTGAYCC			2835R*	GTTCCCAVGWATAWGGTGAYCC	
D	1584F	ACTTCGMBTCACCTCTGCACGT	57	**6**	1584F	ACTTCGMBTCACCTCTGCACGT	57
	2396R	GTCKGCGAGGYGAGGGAGTT			2331R*	GAAGYGTKGAYARGATAGGGGCATT	
E	1190F	AYGCAACCCCCACTGG	51	**7**	1190F*	AYGCAACCCCCACTGG	51
	1797R**	CCAATTTMTGCYTACAGCCTC			1797R	CCAATTTMTGCYTACAGCCTC	

Suppl Table 3: A list of sites at which genetic variants were identified in either Illumina or Nanopore HBV reads. Sites are included that have either (a) >10% variation in Illumina RCA rep1, or (b) variant allele frequency > 10%, both concatemer-association p-values < 0.01, and strand bias p-value > 0.01 in the Nanopore data. The QUAL score is based on a combination of the two concatemer-association p-values (see methods). QUAL scores are capped at 66 as this corresponds to the minimum p-values reported by the Fisher's Exact test.

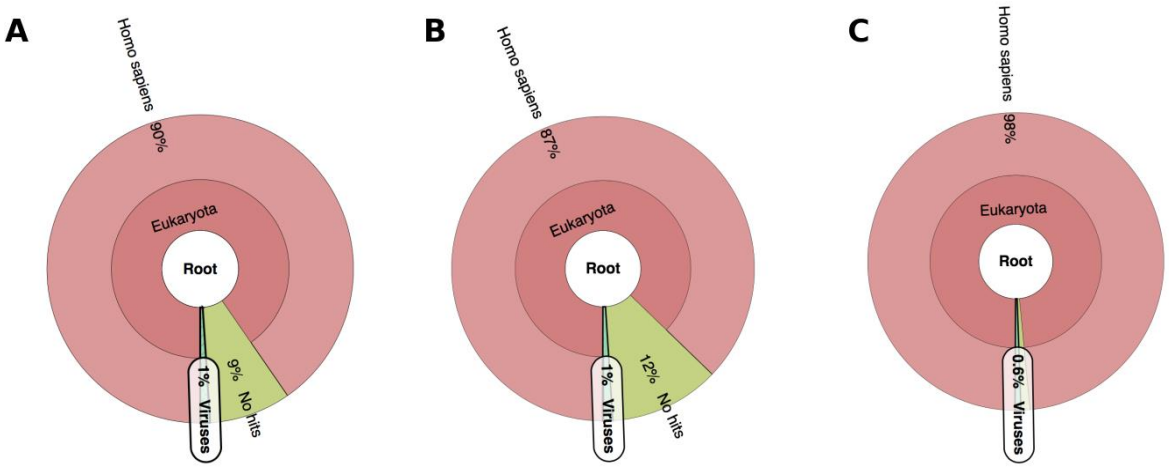
Sample ID	Genome position	Nanopore QUAL score	Variant frequency in Illumina RCA rep 1 (%)
1331	1041	66	17
1331	1054	66	20
1331	1936	66	39
1331	2134	66	25
1348	400	66	5
1348	841	66	8
1348	915	66	11
1348	1425	66	21
1348	2189	66	5

Suppl Fig 1: The density of insert ends along the genome in CL and CL + RCA Illumina sequencing data from HBV sample 1348.



Suppl Fig 2: Krona plots to illustrate the proportion of human vs virus (majority hepatitis B virus) reads in Illumina data derived from all samples.

A: Sample 1331. B: Sample 1332. C: Sample 1348



Suppl Fig 3: Illustration of Nanopore sequence data derived from a mixture of two plasma samples from adults with chronic HBV infection (ID 1331 and 1332, genotypes C and E, respectively), showing classification of 4799 single genome segments as either genotype C and E, and detailed visualisation of 6 unclassified segments.

A: Plot showing the similarity of read segments to genotype C vs E, as assessed by the aligned bases at 335 genotype-discordant sites. Each point represents one of 4805 genome segments contained within concatemeric reads derived from the mixed sample. These segments have been mapped, using bwa mem, to a fasta file containing both a genotype C and genotype E reference sequence, and points are coloured according to which genotype the majority of segments within the concatemer mapped to (red = C, yellow = E). Points are ordered along the x-axis according to read name. The vast majority (4799) segments can be classified as either genotype C (proportion C > 0.8) or E (proportion C < 0.2). No segment has a classification that differs from the majority genotype of the concatemer (as represented by the colour).

B: A representation of the 6 concatemers containing segments that could not be classified as either genotype C or E. Coloured columns represents genotype-discordant sites, arranged in the order they appear along the genome. For each segment, these are coloured as red (match to C/1331 consensus), yellow (match to E/1332 consensus) or grey (match to neither/missing data). Columns on the left hand side indicate the read name, segment start, majority genotype from bwa mapping, and proportion genotype C, where cells containing a proportion between 0.2 and 0.8 are highlighted in green. 4/6 of these segments cover less than 8 genotype-discordant sites, the remaining two appear to show the characteristics of poor quality sequence data, matching to a mixture of gt C, gt E, or neither along the whole length of the segment.

Fig 3A:

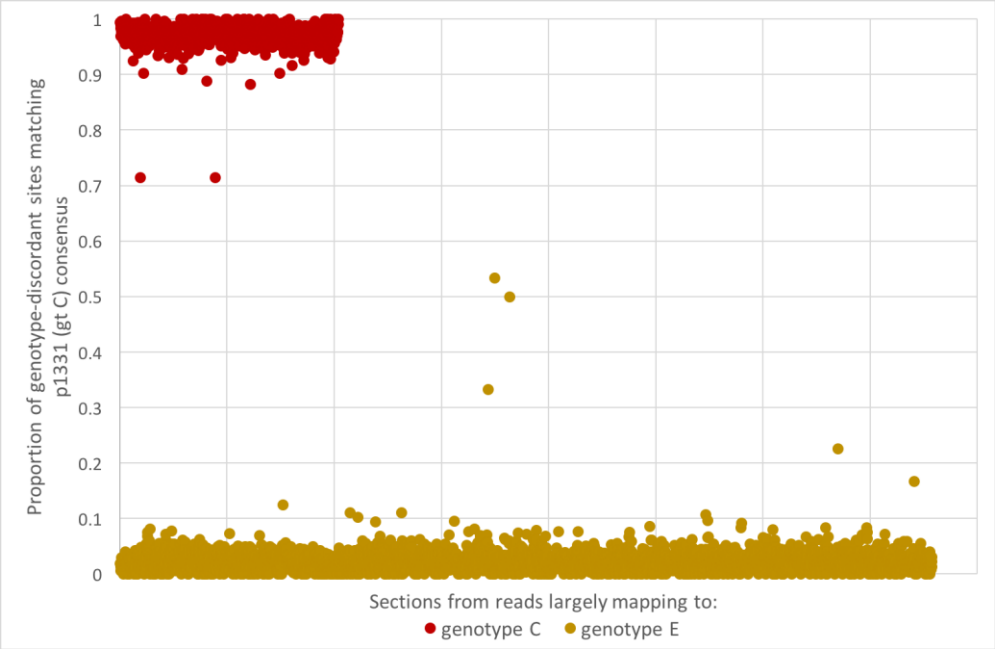
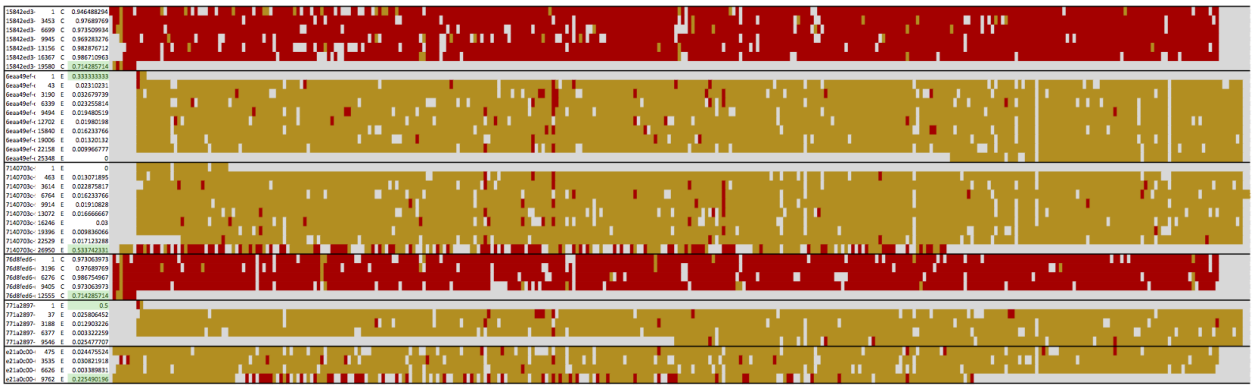
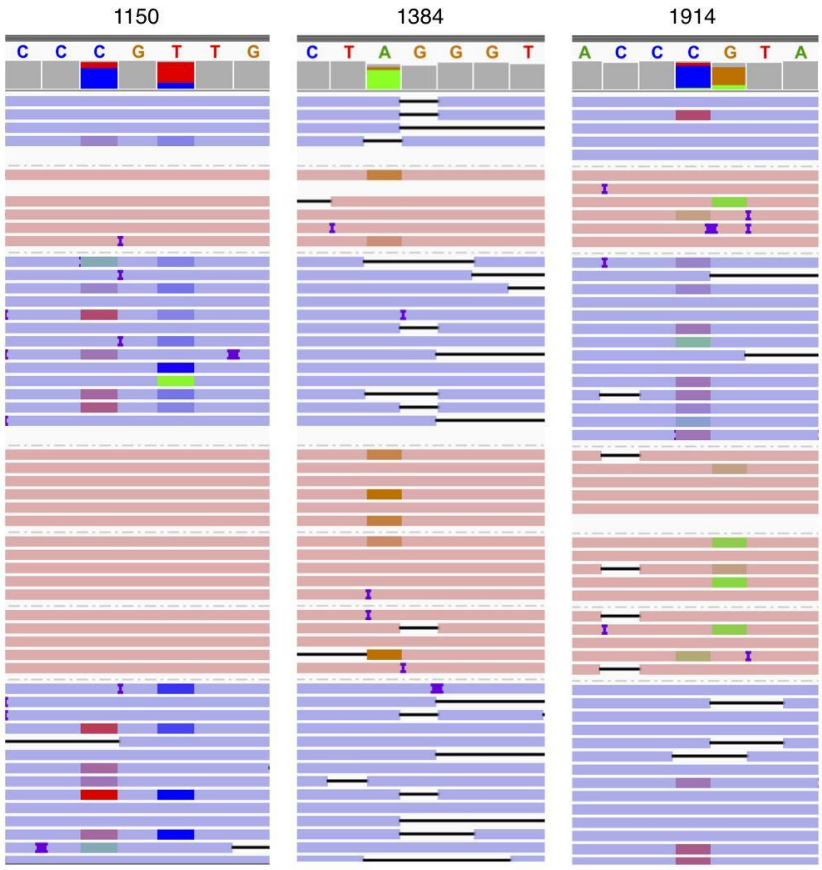

Proportion of genotype-discordant sites matching p1331 (gt C) consensus
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sections from reads largely mapping to:
genotype C
genotype E

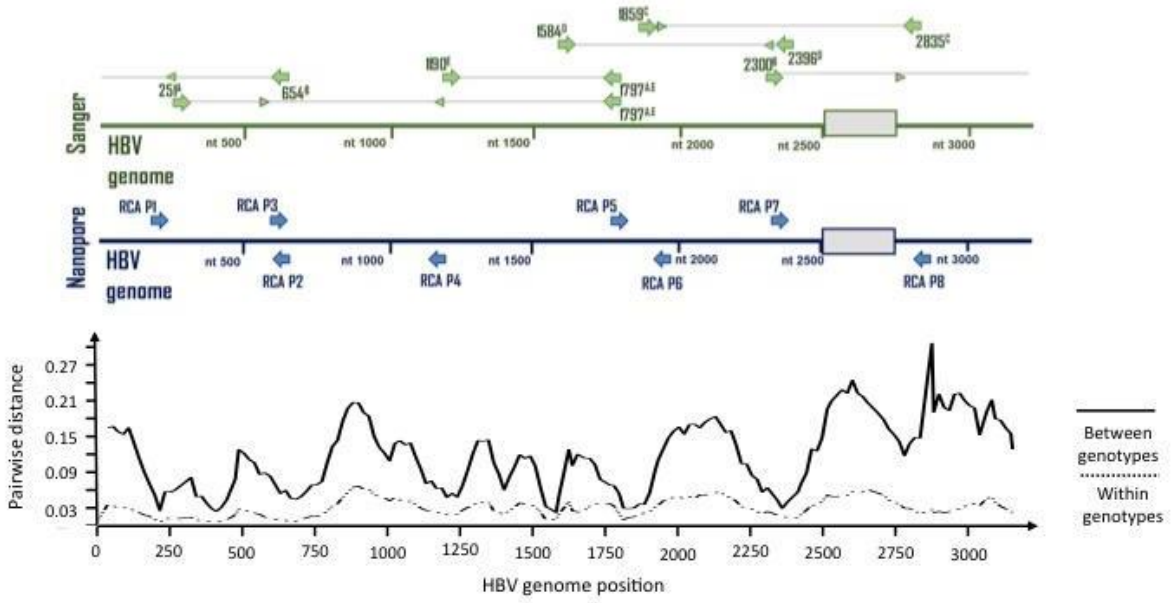
Fig 3B:



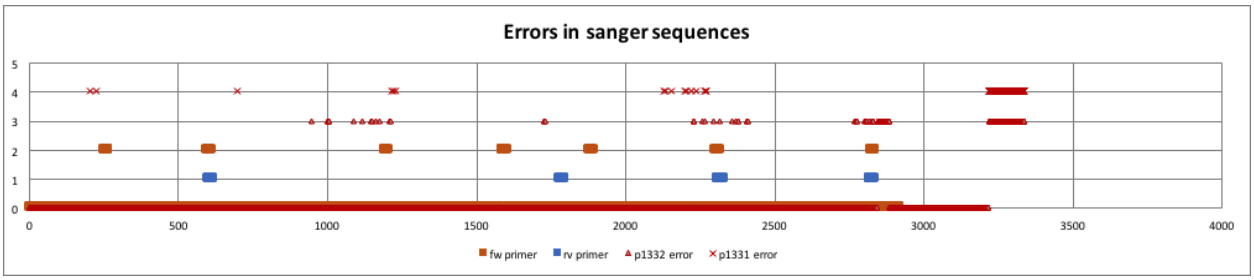
Suppl Fig 4. Examples of kmer-specific errors in Nanopore reads. Snapshots from the Integrative Genomics Viewer (IGV)[4] for sample 1331 are shown. Reads have been aligned to the genotype C reference, the sequence of which is shown at the top of each panel. Coverage is indicated by the grey columns below the reference sequence. For sites with >10% variation, these columns are coloured according the the distribution of bases at that site. Read-sections are grouped according to the concatemers they originated from and coloured by strand (+ = red, - = blue). Insertions, deletions and mismatches are indicated as described in Suppl Fig 1. The three snapshots are centred on positions 1150, 1384 and 1914 (from L to R). In each of the cases shown the variant alleles occur almost exclusively in reads from only one of the strands (at sequence positions 1149, 1151 and 1914 the variant appears in the - strand, while in 1383 and 1915 the variant is only seen in the + strand).



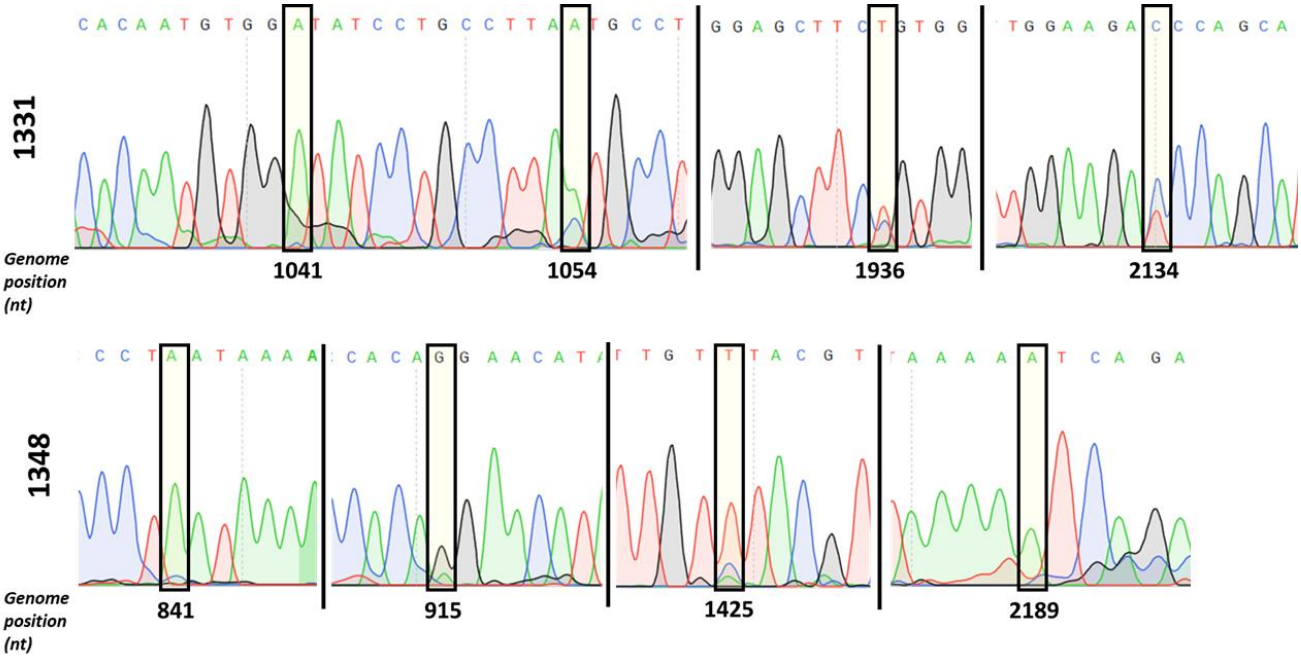
Suppl Fig 5. Primer sequence locations for Sanger sequencing and rolling circle amplification (RCA) mapped onto the HBV genome. Primer locations are indicated with arrows. For the Sanger sequencing primers, triangles have also been used to illustrate the locations of additional primers used for amplification of the inner fragments during the nested PCR (see Suppl Table 2 for more details). Grey boxes indicate a region of the genome, at approximately nt 2500-2700, that was found to have a consistent drop in coverage with all sequencing approaches. A plot of the average pairwise distance of HBV sequences, both within and between genotypes, is also shown to highlight diverse regions of the genome.



Suppl Fig 6: Errors in Sanger sequences relative to sites of sequencing primers, based on consensus generated by Illumina sequences. Errors shown for sequences derived from adults with chronic HBV infection with viral load >10^8 IU/ml, recruited in Oxford (sample ID 1331 and 1332). Derived Sanger sequences for 1331 and 1332 were 3215 bp and 3172 bp in length respectively, with the reduction in length of 1332 largely accounted for by a poorly sequenced region at nt 2843-2882 due to short overlap between two of the overlapping fragments.



Suppl Fig 7: Sites of diversity in Sanger sequence chromatograms from samples 1331 and 1348. Sites of diversity were identified in Nanopore and Illumina sequencing data, as listed in Suppl Table 3 and searched for in Sanger sequences spanning the relevant sites. The sites at nucleotide position 400 in sample 1348 is not shown due to the poor sequencing quality at this site making it difficult to infer if diversity is present at the site. Each site has been highlighted in the region shown and the genome position is cited.



References for supplementary material:

1. Chook JB, Teo WL, Ngeow YF, Tee KK, Ng KP, Mohamed R. Universal Primers for Detection and Sequencing of Hepatitis B Virus Genomes across Genotypes A to G. J Clin Microbiol. 2015;53: 1831–1835.

2. Simmonds P. SSE: a nucleotide and amino acid sequence analysis platform. BMC Res Notes. 2012;5: 50.

3. Garson JA, Grant PR, Ayliffe U, Ferns RB, Tedder RS. Real-time PCR quantitation of hepatitis B virus DNA using automated sample preparation and murine cytomegalovirus internal control. J Virol Methods. 2005;126: 207–213.

4. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. Nat Biotechnol. 2011;29: 24–26.