Insights into blood donor screening for occult hepatitis B virus infection

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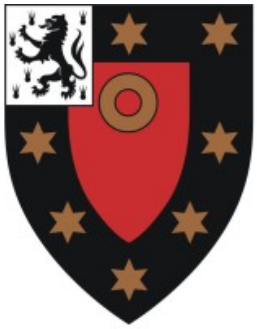

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Table of Contents

Abstract

Occult hepatitis B virus (HBV) infection remains a risk to the blood supply. Prevention of transmission by transfusion requires a numerous set of tests, including surface antigen, anti-core antibodies and HBV DNA. Since anti-core testing was introduced in 2022 in England, the effectiveness of testing and potential alternatives required investigations. This thesis details the development of assays that uncover the existence of donors with HBV DNA viral loads below the limit of detection of commercially available assays; the potential utility (and insensitivity) of next-generation sequencing assays in blood donor screening; the need for a second screening assay to reduce false positivity in anti-core screening assays; and an international survey gained insight into the screening practices for occult HBV infection, effectiveness of screening in preventing transfusion-transmitted infections, and the lack of consensus regarding donor follow-up. These results highlight the complexity of the diagnosis and care of individuals with occult HBV infection, and conclude that while screening strategies are necessary, a more sensitive screening strategy to identify occult HBV infections would improve blood safety even further. Further research could identify alternative biomarkers that could predict the presence of infectious forms of occult HBV, as well as gather insights from donors with occult HBV infection into their diagnosis and follow-up.

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P.S. As advised, I acknowledge the journals where my DPhil work has been published, from which parts of this thesis are derived. References to these publications are provided at the beginning of each chapter, and figures and tables are attributed accordingly, with the consent of my supervisors.

List of publications

- **Fu MX**, Simmonds P, Andreani J, Baklan H, Webster M, Asadi R, Golubchik T, Breuer J, Ijaz S, Ushiro-Lumb I, Brailsford S, Irving WL, Andersson M, Harvala H. Ultrasensitive PCR system for HBV DNA detection: Risk stratification for occult hepatitis B virus infection in English blood donors. J Med Virol. 2023 Oct;95(10):e29144. doi: 10.1002/jmv.29144.
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Other publications, prizes & conferences during the DPhil

Other publications

- **Fu MX**, Lambert G, Cook A, Ndow G, Haddadin Y, Shimakawa Y, Hallett TB, Harvala H, Sicuri E, Lemoine M, Nayagam S. Quality of life in patients with HBV infection: A systematic review and meta-analysis. JHEP Rep. 2025 Jan 8;7(4):101312. doi: 10.1016/j.jhepr.2024.101312.
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- McClure CP, Kean K, Reid K, Mayne R, **Fu MX**, Rajendra P, Gates S, Breuer J, Harvala H, Golubchik T, Tarr AW, Irving WL, Makris M, Simmonds P. Reconstruction of the historic time course of blood-borne virus contamination

of clotting factor concentrates, 1974-1992. J Med Virol. 2024 Jul;96(7):e29774. doi: 10.1002/jmv.29774.

Prizes and Grants

- Race & Sanger Award, British Blood Transfusion Society, 2025
- RCPath William Tong Prize, 2023
- Best Oral Presentation, 1st Annual UK Hepatitis B Virus Meeting, 2023
- European Society for Clinical Virology Travel Grant, 2023 & 2025

Conference Presentations and Courses

- British Blood Transfusion Society Annual Conference, 2025
- 27th European Society for Clinical Virology Annual Meeting, 2025
- 2nd UK HBV Meeting, 2024
- 38th International Society of Blood Transfusion International Congress, 2024
- Wellcome Genome Campus Genomics and Clinical Virology course, 2024
- 1st UK HBV Meeting, 2023
- 25th European Society for Clinical Virology Annual Meeting, 2023
- UK Clinical Virology Network Annual Scientific Conference, 2023
- European Society for Clinical Virology NGS Workshop, 2022

List of figures

below each extraction method. The number of positive control panel samples (n=26) detected by each method is shown. Extraction methods are displayed on the x-axis from smallest to largest sample representation.

- **Figure 8**.[2] Differences in log-transformed estimates of VLs derived from each method with the mean VL for the 17 samples with detectable HBV DNA in all extraction methods. The median and interquartile ranges for each method are displayed, with the continuous dotted line showing the log mean of each sample as 1.0. Both graphs display the methods on the x-axis from smallest to largest sample representation.
- **Figure 9**.[2] Comparing a range of PCR template volumes and the resulting log IU detected across four samples with varying viral loads (VL).
- **Figure 10**.[2] Probit analysis to determine the 95% and 50% detection endpoints of the optimal PCR system, showing the probabilities and the corresponding estimated log (IU) of HBV DNA. The ultrasensitive PCR assay developed following extensive testing validations was used for this analysis, using 5 mL extraction method, PCR-C, and 23.75 µL DNA template in the assay. The additional dotted curves represent the 95% confidence limits. Results of the multiple replicates of sequential 1:2 dilutions of the 4th WHO International Standard for HBV DNA from 45.36 IU to 0.09 IU used for Probit analysis are shown below the plot.
- **Figure 11**.[2] Screening and reference lab testing pathway for NHSBT donations since the introduction of anti-HBc screening in May 2022 (black boxes). 134 of 412 confirmed anti-HBc-positive donations with low anti-HBs levels (<100 IU/L) were assayed by ultrasensitive PCR (blue boxes). Extrapolation of HBV DNA detection rate to all 412 HBsAg-negative, anti-HBc-positive, and anti-HBs<100 IU/L donations is shown in grey boxes.
- **Figure 12.**[2] a) Comparisons of anti-HBs, anti-HBc, and anti-HBe reactivity ratios (based on platform) between DNA-positive donors and DNA-negative donors, where error bars represent medians and interquartile ranges. The dotted blue lines represent the protective cut-off value for anti-HBs (10 IU/L) and anti-HBe (1) where values below are considered negative. * denotes $p \leq 0.05$, *** denotes $p \leq 0.001$ assessed by Mann-Whitney U tests, and ns denotes non-significance; b) Receiver operating characteristic curve (ROC) analysis predicting the anti-HBc and anti-HBs ratio thresholds that would be most sensitive and specific in identifying DNA-positive donations. The dotted line represents the line of identity; c) Anti-HBc S/CO ratios obtained from ROC curve analysis with a range of sensitivities.
- **Figure 13.**[3] Comparison of HBV viral loads obtained from four concentration methods for four samples: standard extraction using 0.2 mL of plasma (Method 1) and 5 mL of plasma (Method 2), polyethylene glycol (PEG) precipitation using 10 mL (Method 3) and 20 mL (Method 4) of plasma. The error bars indicate the mean and standard error of the mean from three PCR replicate measurements. The volume of plasma sample used, PEG, resuspension volumes, extraction volumes, elution volumes and effective test volumes ([volume of input plasma] / [elution volume] * [PCR template volume]) are displayed below the plot. Neg = sample not detected by this method, which could be explained by potential difficulties in large pellet resuspension with the doubling of input volumes than recommended in the manufacturer's protocol.

- **Figure 14.**[3] Comparison of viral loads obtained from the five concentration methods: standard extraction without concentration using 0.2 mL (Method 1) and 5 mL of plasma (Method 2), polyethylene glycol (PEG) precipitation using 10 mL (Method 3) and 20 mL (Method 4) of plasma, and ultracentrifugation using 35 mL of plasma (Method 5): A) Comparison of calculated viral loads (normalised to input plasma volume) for each methods, with log-log non-linear regression lines shown and axes drawn to the same scales for each plot. B) Comparison between Methods 1 to 4 (samples were concentrated, extracted, and tested concurrently for Methods 2 to 4) showing calculated viral loads (normalised to input plasma volume) for the set of 23 samples, where the lines indicate the median [interquartile range] for each method. The volume of PEG, resuspension volumes, extraction volumes, elution volumes and effective test volumes ([volume of input plasma] / [elution volume] * [PCR template volume]) are displayed below the plot. *P*-values were obtained from the Friedman test with Dunn's multiple comparisons test. Lighter-coloured dots indicate measurements from 9.5 µL extracts instead of 2 µL due to limited extract, as measurements were initially performed with 9.5 µL extracts. IU/mL = international units per millilitre.
- **Figure 15**.[3] Comparison between Methods 0 to 4 (samples were concentrated and extracted concurrently for Methods 1 to 3 and were tested concurrently for Methods 1 to 4) showing viral loads (normalised to input plasma volume) for the 12 samples with the lowest viral loads, where the lines indicate the median [interquartile range] for each method. The volume of PEG, resuspension volumes, extraction volumes, and elution volumes are displayed below the plot. *P*-values were obtained from the Friedman test with Dunn's multiple comparisons test. Lighter-coloured dots indicate measurements from 9.5 µL extracts instead of 2 µL due to limited extract, as measurements were initially performed with 9.5 µL extracts. IU/mL = international units per millilitre.
- **Figure 16**.[3] Read depth plots across whole viral genomes for the samples run through the next-generation sequencing protocol. Plots a) to c) show the comparison of genome coverage for HBV and HHV8 detected in samples by both 5 mL (Method 2) and PEG methods (Method 3 or 4), whilst d) and e) show genome coverage for HBV and EBV detected in samples using only the PEG method. Anellovirus coverage is not shown due to viral heterogeneity. HBV = hepatitis B virus; PEG = polyethylene glycol; HHV = human herpesvirus; EBV = Epstein-Barr virus.
- **Figure 17**.[4] Relationship between viral loads and HBV-specific bases read for each group of methods on a common log scale, when majority consensus bases were called at a threshold of 1 base per nucleotide site. Pink indicates contaminating sequences, including where HBV sequences were obtained for the negative HBV10 sample on the y-axis. Samples not detected are found on the x-axis. Spearman's correlation test deduced the significance of the association between viral loads and base counts (R^2 value and *p*-values listed in boxes). HBV: hepatitis B virus; MTG: untargeted metagenomics; TAC: target capture; ONT: Oxford Nanopore Technologies.
- **Figure 18**.[4] Relationship between viral loads and breadth coverage of HBV assembled sequences for each group of methods on a common axes scale, when majority consensus bases were called at a threshold of 1 base per nucleotide site. Pink indicates contaminating sequences, including where HBV sequences

were obtained for the negative HBV10 sample on the y-axis. Samples not detected are found on the x-axis. Spearman's correlation test deduced the significance of the association between viral loads and genome coverage (R^2 value and *p*-values listed in boxes). HBV: hepatitis B virus; MTG: untargeted metagenomics; TAC: target capture; ONT: Oxford Nanopore Technologies.

- **Figure 19**.[4] Normalised mean read depth of the HBV genome for each of the sequencing methods. The normalised mean read depth was calculated as the number of bases at each genome position as a proportion of the total reads of the sequence, then multiplied by the total number of sites, where the expected mean value was one base per site. The eleven highest viral load samples were included when detected, and only true-positive sequences with more than 1000 total HBV-specific bases read were included; the number of samples from each protocol that were included is indicated in each plot's key. Genome positions were based on the D00330 reference sequence. A genome diagram of HBV, drawn to the x-axis scale, shows gene positions in shaded boxes and regulatory regions in unshaded boxes. TAC: target capture; MTG: untargeted metagenomics.
- **Figure 20**.[4] Relationship between viral loads and mean Shannon entropy values for polymorphic sites for each group of methods on a common axes scale, when majority consensus bases were called at a threshold of 1 base per nucleotide site. Pink indicates contaminating sequences, including where HBV sequences were obtained for the negative HBV10 sample on the y-axis. Samples not detected are found on the x-axis. Spearman's correlation test deduced the significance of the association between viral loads and entropy values (R^2 value and *p*-values listed in boxes). HBV: hepatitis B virus; MTG: untargeted metagenomics; TAC: target capture; ONT: Oxford Nanopore Technologies.
- **Figure 21**.[4] Comparison of a) time taken, and b) cost based on the total time and costs for a protocol-specific representative number of samples included in a typical run. For Figure 5a, undotted bars to the left of the plot show per-person labour time per sample, whereas dotted bars to the right show the waiting time per sample, which may include machine running times. For clinical purposes, PCR-1 is normally run overnight on the MinIon flow cell (average of 16 hours), but was run for 72 hours for this study. Costs were standardised using a 1:0.8959:0.7531 USD:EUR:GBP exchange rate, as of May 18, 2025, and total costs were rounded to the nearest ten. USD: United States Dollar; EUR: Euro; GBP: Great British Pound; QC: quality control; TAC: target capture; MTG: untargeted metagenomics.
- **Figure 22**.[5] Determination of the optimal concentrations of blocking recombinant HBV core antigen (rHBcAg) on the A) in-house anti-HBc IgG assay and the B) Murex anti-HBc assay. 1:2 serial dilutions of rHBcAg were used with anti-HBc positive samples (anti-HBc reactive in both screening assays, anti-HBe positive), initially indeterminate samples (anti-HBc reactive in both screening assays, anti-HBe negative), and negative controls (anti-HBc negative in the Murex assay, anti-HBe negative). The median [range] blocking percentages are displayed in plot a) for each category of samples, where rHBcAg concentrations of 0.25 μg/mL and 1 μg/mL block ≥50% anti-HBc reactivity and block the most signals, respectively. For plot a), twenty confirmed anti-HBc positive controls (comprising 17 HBsAg-positive samples from the HBV-positive control panel previously described[2] and three anti-HBe

positive samples from the current study), thirteen anti-HBc indeterminates and five anti-HBc negative controls (comprising four anti-HBc initial reactive but negative in the second assay plus a negative pool of sera consisting of three donations tested negative for HBV markers) were used to develop the blocking assay. Plot b) displays the blocking percentages for each sample, determined using 1:2 serial dilutions of the commercial rHBcAg from 16 µg/mL to 0.25 µg/mL, together with a panel of 5 anti-HBc positive controls, three indeterminates, and three negative controls.

- **Figure 23**.[5] Determination of the capacity of rHBcAg of genotype D to block anti-HBc antibodies induced by various HBV genotypes using samples from an HBV-positive control panel. Included were four samples of genotype A, two samples of genotype B, three samples of genotype C, five samples of genotype D, and three samples of genotype E. The median [range] blocking percentages of each genotype are displayed. The chosen blocking concentrations are written above the relevant points.
- **Figure 24**.[5] Determination of the optimal urea concentration with serial dilutions of urea in phosphate-buffered saline Tween (PBST) from 10M to 2M and ten HBsAg-positive samples from the control panel and one IgM-positive sample. Error bars represent the means [standard deviations]. A concentration of 6M provided the highest difference in avidity between the chronic and acute HBV samples.
- **Figure 25**.[5] Determination of the linear range of optical density (OD) absorbance values that produce consistent avidity indices between 1:2 dilutions of nine chronic and one acute HBV samples (where the data points at the far right represent the initial dilutions at the upper limit of detection, and each subsequent data point to the left represents sequential 1:2 dilutions). The avidity index for each sample at each dilution is shown.
- **Figure 26**.[5] Correlation of log IU/mL values from the in-house assay with the Architect and Murex screening log ratios.
- **Figure 27.**[5] a) Median [interquartile range] blocking percentages in all 173 anti-HBc positive controls (147 anti-HBc reactive in both screening assays, anti-HBe positive and 26 samples from the HBV control panel) and anti-HBc negative controls (anti-HBc negative in the second screening assay, anti-HBe negative, anti-HBs negative) tested in the in-house assay. Thirty-two of 51 negative controls were anti-HBc negative in the in-house assay, and the other 19 controls underwent blocking. The blocking percentages of 23 anti-HBc positive controls tested in the Murex assay are also shown; b) Comparison of Architect, Murex and in-house anti-HBc ratios between positive and negative controls, showing all quartiles; c) Comparisons of the median [interquartile range] Architect, Murex and in-house anti-HBc ratios between donor samples that were anti-HBc blocked (n=310) and samples that were either not blocked or were anti-HBc negative (n=87). The dotted lines represent the 1.0 cut-off value for the anti-HBc screening assays. **** indicates $p<0.0001$ from Mann-Whitney U tests.
- **Figure 28.**[5] The flow diagram shows the delineation of anti-HBc reactivity based on extensive testing of the 397 HBsAg-negative and anti-HBc initial reactive samples on the Architect assay. Testing included the Murex anti-HBc assay, anti-HBe testing, in-house anti-HBc blocking assay, blocking on the Murex assay, and the anti-HBc LIASION test. The cumulative positive predictive value (PPV) and negative predictive value (NPV) of the five anti-

HBc assays to the true-positive and false-positive samples in the 'Conclusion' are shown in the top-right of each anti-HBc assay, where minimal changes are observed after the Murex assay.

- **Figure 29.**[5] Correlation between avidity and anti-HBc a) Architect S/CO ratio (r^2=0.196), b) anti-HBc Murex S/CO ratio (r^2=0.109), and c) anti-HBc IU/mL from in-house assay (r^2=0.006), for all 99 samples tested for avidity and blocked. The dashed lines separate each anti-HBc ratio into a cut-off value for samples with lower avidity.
- **Figure 30**.[5] Comparisons of median [interquartile range] initial anti-HBc signal to cut-off (S/CO) ratios between the 397 donor samples categorised by anti-HBc genuine positivity, false positivity, and indeterminate following the extensive testing detailed in Figure 2. The true-positive samples are further classified by anti-HBe status on the left hand of the graph, separated by a dotted line. The proportion of minority ethnic group donors for each category of donors is indicated, as well as from all donors in 2021. The Mann Whitney-U test compared anti-HBe status (darker coloured line), whilst the Kruskal-Willis test with post-Hoc Dunn's compared between positivity categories (light coloured lines), and Chi-squared tests compared ethnic groups, where *** denotes p<0.001 and **** denotes p<0.0001.
- **Figure 31**.[5] Receiver operator characteristic (ROC) analysis to investigate Architect S/CO ratio (green) and anti-HBe ratio (purple) thresholds that may predict anti-HBc true positivity. For this analysis, anti-HBc true positives and true negatives (false positives) were utilised from this study's 397 samples, excluding the five indeterminate samples previously shown. The 90%/95% sensitivity and specificity threshold values for anti-HBc and anti-HBe are shown in the tables below the plot and 100% specificity thresholds for anti-HBc positivity.
- **Figure 32.**[6] Geographical locations of survey respondents, showing whether countries have regional or national respondents and delineating HBV screening strategies using differing colours. A summary table of anti-HBc and HBV NAT use for blood donation screening by participating respondents (n=30) and countries (n=25) is shown below the map. Blue indicates countries using anti-HBc and HBV NAT testing, purple indicates countries utilising anti-HBc testing without HBV NAT testing, and yellow indicates countries utilising HBV NAT testing without anti-HBc testing. Green indicates countries that do not perform either anti-HBc or HBV NAT testing of blood donations. This map was made with mapchart.net.
- **Figure 33**.[6] Simple linear regression plots of country HBsAg positivity with a) anti-HBc positivity rate in all donors, new donors, and repeat donors for all countries that perform universal anti-HBc screening, and b) each country's reported OBI prevalence for all countries that screen using NAT.

List of tables

- **Table 9.**[2] Comparison of HBV DNA detection in IU between the two commercial methods at NHSBT England and the ultrasensitive PCR, showing the 95% limits of detection and effective test volumes (negating the dead volumes where applicable for the commercial assays – Grifols input 0.800 mL and dead volume 0.300 mL; Roche input 1.300 mL and dead volume 0.450 mL).
- **Table 10**.[4] Protocol details of the NGS protocols analysed, including the sequencing workflows and analysis pipelines (until the point of genome assembly) for each method. Effective test volume was calculated as [library prep input volume]/[extraction output volume]*[extraction input volume], where TAC-A, TAC-B and MTG-B also accounted for reductions in effective test volume through cDNA synthesis, and PCR volumes were also accounted for in PCR-based protocols. Numerical superscripts next to method identifiers indicate where the same laboratory performed the protocols.
- **Table 11**.[4] Details of primers used for sequencing of HBV amplicons, indicating the start and end nucleotide positions of the amplified regions. Outer primer sets are provided in the above cell, while inner primers are indicated in the below cell. Nucleotide positions are numbered according to the D00330.1 reference sequence.
- **Table 12**.[4] Overview of the detection of HBV in sample sets, when majority consensus bases were called at a threshold of 1 base per nucleotide site. The percentage genome completeness is given in each cell. The left-hand side of the table with blue headers shows the sequences obtained from the NGS methods, whilst the right-hand side (calamine colour) shows the sequences obtained by Sanger sequencing (length of S amplicon = 395 nucleotides; SP = 973 nucleotides; XC = 698 nucleotides). Within-sample consensus sequences were assembled for each sample from the individual sequences of the NGS methods that had around 100% genome coverage and were genetically similar, which were assigned to HBV genotypes. The percentage genetic relatedness of the sequences from each method was compared to the within-sample consensus sequence, where assembled sequences similar to the within-sample consensus were shaded on a grayscale, and different strains (differing in >5% nucleotide sequence from the within-sample consensus) were shaded in a pink scale. The absence of sequences was shaded in black. Where within-sample consensus sequences could not be assembled (i.e. no two complete sequences were similar), genotypes were defined from Sanger sequences, and the NGS sequences were instead compared to one another. Samples are ranked from highest to lowest geometric mean viral load. For TAC-B: library preparation failed for sample HBV13; samples HBV4, HBV9, and HBV19 had reads of poor quality for the original pipeline to process, so the mean base quality was dropped below 1.0. #: For PCR-1, a negative water control was tested instead of the negative plasma control. Alphabetical superscripts (a, b, c) represent identical sequences. Numerical superscripts next to method identifiers indicate where the same laboratory performed the protocols.
- **Table 13**.[4] Overview of the detection of HBV in sample sets, when majority consensus bases were called at a threshold of 10 bases per nucleotide site. Otherwise, same legend as Table 12.
- **Table 14**.[4] Detection of other viruses by the targeted capture and untargeted metagenomics methods, showing deduplicated read counts (genome coverage %). Results reported for TAC-C are for viruses where at least 10 reads

were obtained. More than one TTV species may have been identified for MTG-A, but the most abundant was included. The furthest right column shows the PCR results, where PCR was performed for all samples when two or more methods detected a virus in the same sample. These PCRs were specific for alpha, beta and gamma-torquevirus, Merkel cell polyomavirus, and herpesviruses. '-' denotes where the method did not detect the virus, '=' denotes where the PCR was not performed. A numerical superscript indicates where the same laboratory performed the protocols. HHV: human herpesvirus; MCPyV: Merkel cell polyomavirus; AV: anellovirus; TTV: torquetenovirus; EBV: Epstein-Barr virus; KSHV: Kaposi's sarcoma-associated herpesvirus; JCPyV: John Cunningham virus.

- **Table 15.**[4] Specific criteria used by protocols to call majority bases and positive detection.
- **Table 16**. Overview of the mutations observed in global consensus sequences obtained by NGS for OBI samples in the sample panel. Where mutations were observed in OBI in previous studies, these are referenced. SHB = small surface region; RT = reverse transcriptase domain. Mutations were identified through the geno2pheno software.
- **Table 17.**[5] Serological and demographic characteristics of the eleven inconclusive samples identified from the in-house blocking assay. Serological and demographic characteristics of the eleven inconclusive samples identified from the in-house blocking assay. Samples are labelled according to their final status: Murex blocking assay +/- Monalisa assay (Pos), not blocked on the Murex and negative on Monalisa (Ind), Murex-negative and positivity on Monalisa only (Ind), and Murex-negative and Monalisa-positive (Neg). Underlined values indicate positive values according to the assay protocols. Average ratios are shown in the table. NA indicates where the blocking and avidity assays were not done since anti-HBc was negative on the assay. *Where anti-HBs titres were >1000 IU/L, 2000 IU/L was used as an approximation to obtain average values.
- **Table 18.**[5] Comparisons of the proportion of ethnic groups between anti-HBc positive donors (n=981) and anti-HBc negative donors (n=794,861) for all rare blood types and each Rh blood type: R2R2, Ro and rr. The number of donors in each ethnic group for each blood type is shown, as well as the proportion of those donors representing all donors in that ethnic group for anti-HBc positive or negative donors. Fisher's exact tests were used to compute p-values.
- **Table 19.**[6] Demographic data for blood establishments that perform universal anti-HBc screening (n=16). For the country column, shaded cells represent respondents with regional data, whilst unshaded cells represent countries with national-level data. For the 95% LOD column, italicised values represent values obtained from the manufacturer's report, and non-italicized values represent values respondents reported but did not refer to the manufacturer. NA represents 'not applicable'. HBV NAT = hepatitis B virus nucleic acid testing; LOD = limit of detection; OBI = occult hepatitis B virus infection; FFP = fresh frozen plasma.
- **Table 20.**[6] Number and proportion of anti-HBc positive donors, including those with OBI, for the survey respondents that perform universal anti-HBc screening, showing confirmation and deferral strategies applied in 2022 (n=16). Confirmation of anti-HBc positivity was performed on the donation sample

unless specified as ‘Repeat next donation’, in which case confirmation was performed on a second specimen. Shaded cells mean ‘not applicable’.

- **Table 21.**[6] Demographic data for blood establishments that perform HBV NAT screening of all blood donations but do not perform anti-HBc screening on all donations (n=10). Some blood establishments perform targeted anti-HBc screening: the blood establishments that provided this information are indicated, but this does not necessarily mean that the other blood establishments did not utilise a similar strategy. For the country column, shaded cells represent respondents with regional data, whilst unshaded cells represent countries with national-level data. For the 95% LOD column, italicised values represent values obtained from the manufacturer’s report, and non-italicized values represent values respondents reported but did not refer to the manufacturer.
- **Table 22.**[6] Follow-up of anti-HBc positive and OBI donors amongst survey respondents.
- **Table 23**.[1] Summary of the challenges and needs of blood biomarkers of transfusion-transmitted occult hepatitis B virus infection (OBI) currently utilised in diagnostic practice. HBsAg, hepatitis B surface antigen; IU/mL, international units per millilitre; anti-HBs, antibodies against surface antigen; anti-HBc, antibodies against core antigen; MP-NAT, mini-pool nucleic acid testing; ID-NAT, individual donation nucleic acid testing; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus.

List of abbreviations

AI	Avidity index
Anti-HBc	Hepatitis B virus anti-core antibodies
Anti-HBe	Hepatitis B virus anti-e antibodies
Anti-HBs	Hepatitis B virus anti-surface antibodies
cccDNA	Covalently closed circular DNA
DNeasy	DNeasy Blood and Tissue Kit
EBV	Epstein-Barr virus
FFP	Fresh frozen plasma
HBeAg	Hepatitis B virus e-antigen
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HHV	Human herpesvirus
HPyV6	Human polyomavirus 6
ID-NAT	Individual donation nucleic acid testing
ISBT	International Society of Blood Transfusion
JCPyV	JC polyomavirus
Kb	Kilobases
LLOD	Lower limit of detection
LMICs	Low-to-middle-income countries
LOD	Limit of detection
MCPyV	Merkel cell polyomavirus
MP-NAT	Mini-pool nucleic acid testing

MTG	Untargeted metagenomics
NAT	Nucleic acid testing
NGS	Next-generation sequencing
NHSBT	National Health Service Blood and Transplant
OBI	Occult hepatitis B virus infection
OD	Optical density
ONT	Oxford Nanopore Technologies
ORF	Open reading frame
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween
PEG	Polyethylene glycol
pgRNA	Pre-genomic RNA
QIAamp	QIAamp DNA Blood Mini Kit
RBC	Red blood cell
ROC	Receiver operating characteristic
Roche2.5	Roche High Pure Viral Nucleic Acid Large Volume Kit using 2.5 mL extraction volume
Roche5	Roche High Pure Viral Nucleic Acid Large Volume Kit using 5 mL extraction volume
SD	Standard deviation
rcDNA	Relaxed circular DNA
rHBcAg	Recombinant HBV core antigen
SaBTO	Standing Advisory Committee on the Safety of Blood, Tissues, and Organs
TAC	Targeted capture next-generation sequencing

TTV	Torque teno virus
UKHSA	United Kingdom Health Security Agency
VL	Viral load
WGS	Whole genome sequencing
Zymo	Zymo Quick-DNA/RNA Viral Kit
Zymo96	Zymo Quick-DNA/RNA Viral 96 Kit

General introduction

Parts of this chapter are based on the published papers:

Fu MX, Simmonds P, Andersson M, Harvala H. Biomarkers of transfusion transmitted occult hepatitis B virus infection: Where are we and what next? Rev Med Virol. 2024 Mar;34(2):e2525. doi: 10.1002/rmv.2525.

Fu MX, Elsharkawy A, Healy B, et al. Occult hepatitis B virus infection: risk for a blood supply, but how about individuals' health? EClinicalMedicine. 2025 Feb 1;81:103095. doi: 10.1016/j.eclinm.2025.103095.

Fu MX, Simmonds P, Andreani J, et al. Ultrasensitive PCR system for HBV DNA detection: Risk stratification for occult hepatitis B virus infection in English blood donors. J Med Virol. 2023 Oct;95(10):e29144. doi: 10.1002/jmv.29144.

Hepatitis B virus

Hepatitis B virus (HBV) infection constitutes a major global health burden, with an estimated 296 million people chronically infected in 2019[7]. This DNA virus is highly endemic in geographical areas such as sub-Saharan Africa, South-East Asia, China, and South America[8]. HBV infection can be acquired through various routes, including vertical transmission, sexual contact, and unsafe injections[8]. HBV is also the most prevalent transfusion-transmitted viral infection despite screening being implemented[9].

Structure, genomic organisation and life cycle

HBV is a small, enveloped DNA virus classified within the *Orthohepadnavirus* genus of the *Hepadnaviridae* family[10]. The HBV virion, also known as the Dane particle, is approximately 42 nm in diameter and consists of a nucleocapsid core surrounded by a lipid envelope that is embedded with surface antigens (HBsAg). The HBV genome is a partially double-stranded, circular DNA of approximately 3.2 kilobases (kb) in length, encoding four overlapping open reading frames (ORFs): S (surface proteins: pre-S1, pre-S2, and S regions), C (core and precore proteins), P (polymerase), and X (HBx regulatory protein)[11].

The life cycle of HBV is unique among DNA viruses in that it replicates via reverse transcription of an RNA intermediate. HBV first attaches to hepatocytes via interactions between the pre-S1 domain of the L-HBsAg and the sodium taurocholate co-transporting polypeptide receptor on the hepatocyte (Figure 1)[12]. Initial low-affinity binding is mediated by heparan sulphate proteoglycans. HBV then enters the hepatocyte via endocytosis, where the nucleocapsid is released into the cytoplasm. Microtubules aid the transport of the capsid to the nucleus, where the relaxed circular (rc)DNA is released. The rcDNA undergoes repair in the nucleus by host cellular enzymes to form covalently closed circular DNA (cccDNA). The stable episomal form of cccDNA acts as the transcriptional template for all viral RNAs. Using host RNA polymerase II, the cccDNA is transcribed into five major RNA species, including the 3.5 kb pregenomic RNA (pgRNA). Transcribed viral RNAs are exported to the cytoplasm, where translation and the highly error-prone reverse transcription of the pgRNA occur within the nucleocapsid. The newly synthesised rcDNA can either be recycled to maintain the cccDNA pool or be enveloped and secreted as progeny virions.

In addition to complete virions, HBV-infected cells also release non-infectious subviral particles of HBsAg[13].

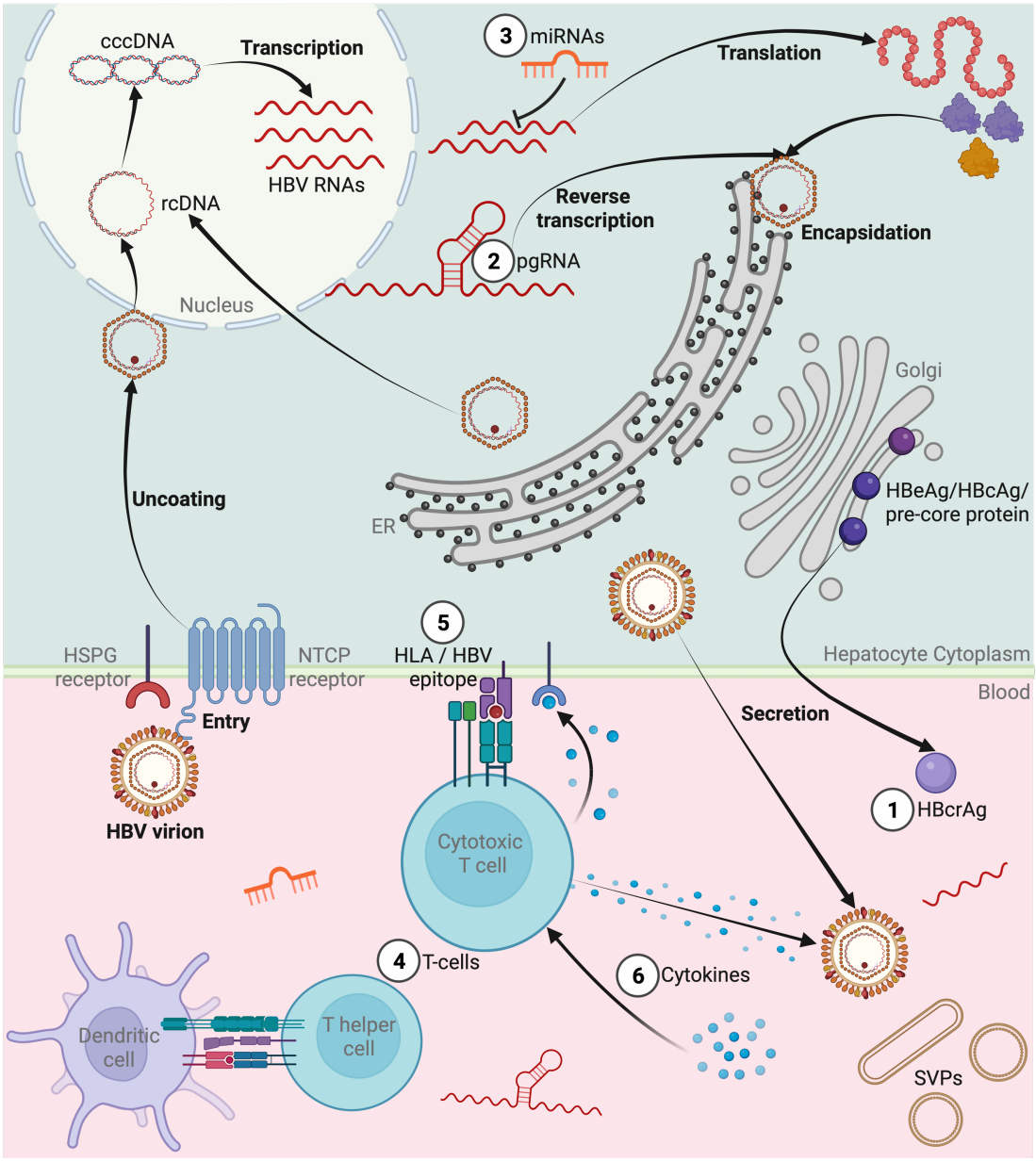


Figure 1[1]. The life cycle of hepatitis B virus (HBV), showing the various novel markers highlighted in the synthesis chapter: (1) HBcrAg, HBV core-related antigen; (2) pre-genomic RNA (pgRNA), pregenomic RNA; (3) miRNAs, microRNAs; (4) T-cells; and (5) HLA, human leucocyte antigen. cccDNA, covalently closed circular DNA; ER, endoplasmic reticulum; HBeAg, HBV e antigen; HSPG, heparan sulphate proteoglycan; IFN-γ, interferon-gamma; NTCP, sodium taurocholate co-transporting

peptide; rcDNA, relaxed circular DNA; SVPs, sub-viral particles. The figure was devised and created by Michael Fu on Biorender.com.

Evolution and genotypes

HBV is classified into ten genotypes (A–J), each differing by more than 8% at the nucleotide level. These genotypes exhibit varying geographic distributions and disease progression, as well as differences in treatment response and the risk of complications. From the more common genotypes, genotype A is prevalent in North America, Africa, and Western Europe. Genotypes B and C are dominant in Asia, genotype D is dominant in the Mediterranean and Middle East, and genotype E is dominant in West Africa. Rarer genotypes include genotype F, indigenous to the Americas; genotype G is globally dispersed; genotype H is found in the Americas and Asia, and genotype I is predominantly in Southeast Asia. Recombination and subgenotypes further contribute to HBV diversity[14].

Mutations

Due to its error-prone reverse transcriptase, HBV is prone to mutations. Common clinically relevant mutations include pre-core and basal core promoter variants, which affect HBV e-antigen (HBeAg) expression and disease phenotype[15]. Mutations in the polymerase gene can confer resistance to nucleos(t)ide analogues, while changes in the S gene may lead to immune escape variants, with implications for diagnostic failure and vaccine efficacy[15]. S gene mutations also play a role in occult hepatitis B virus infection (OBI), although most OBI infections lack antigenic changes. Mutations in the S gene, particularly in the "a" determinant region of the surface antigen, are among the major molecular mechanisms proposed for this phenomenon. These may alter the antigenic structure of HBsAg, leading to escape from immune recognition, or impair the expression or secretion of HBsAg, resulting in false-negative or undetectable results in standard diagnostic assays[1].

Natural history of HBV infection

The natural history of HBV infection is dynamic and can be influenced by factors such as age, immune status, infecting genotype, and coinfections. The clinical course may range from acute self-limiting hepatitis to chronic HBV infection with progressive liver disease and risk of hepatocellular carcinoma (HCC)[16].

1. Acute infection. HBV infection acquired in adults typically results in acute asymptomatic hepatitis with spontaneous seroconversion of HBsAg to anti-HBs in >90% of immunocompetent adults. However, a significant proportion of neonates and children progress to chronic HBV infection.
2. Chronic HBV infection is defined by the persistence of HBsAg for at least six months, characterised by initially high HBV DNA levels and elevated HBeAg levels, which persist until the host immune response becomes more active, leading to hepatic necroinflammation and fluctuating HBV DNA levels (Figure 2). Following seroconversion of HBeAg, HBV infection enters a low replicative phase with undetectable or low HBV DNA levels and minimal liver inflammation. A small proportion of patients (around 1% per year) may lose HBsAg and develop anti-surface antibodies (anti-HBs), a state known as 'functional cure'. However, cccDNA persists in the liver and reactivation remains possible under immunosuppression. Chronic HBV infection can lead to progressive liver damage, including fibrosis, cirrhosis, and HCC. Whilst over 95% of immunocompetent adults achieve functional cure with sustained HBsAg loss following acute self-limiting infection, functional cure in chronic HBV infection remains uncommon, occurring in only single-digit percentages even with long-term antiviral therapy.

3. OBI represents a hidden reservoir of HBV, which may result from very low-level HBV replication, mutations in the S gene that impair HBsAg expression or antigenicity, or host immune control that suppresses replication. In the natural history of HBV infection, OBI may represent a stage during recovery from acute or chronic HBV infection, or following spontaneous or treatment-induced HBsAg loss, or in individuals with previous HBV exposure with persisting cccDNA[17].

Occult hepatitis B virus infection

OBI is a challenging clinical entity that is underdiagnosed. OBI is formally defined as the detectability of replication-competent cccDNA in the liver with or without the presence of circulating HBV DNA, but when HBsAg is undetectable by currently available assays[17]. However, no established markers predict the presence of cccDNA in the liver independently of the detection of circulating HBV DNA in plasma[1]. Laboratory diagnosis of OBI based on plasma samples alone is thus somewhat dependent on the (in)sensitivity of the serological assay used for HBsAg detection and, in the absence of a liver biopsy sample, the sensitivity of the assays used to detect HBV DNA in plasma. In addition, the tendency of some OBI carriers to have HBV DNA levels that fluctuate above and below the lower limit of laboratory detection introduces both the frequency of testing and an element of chance into the likelihood of identifying OBI (Figure 2).

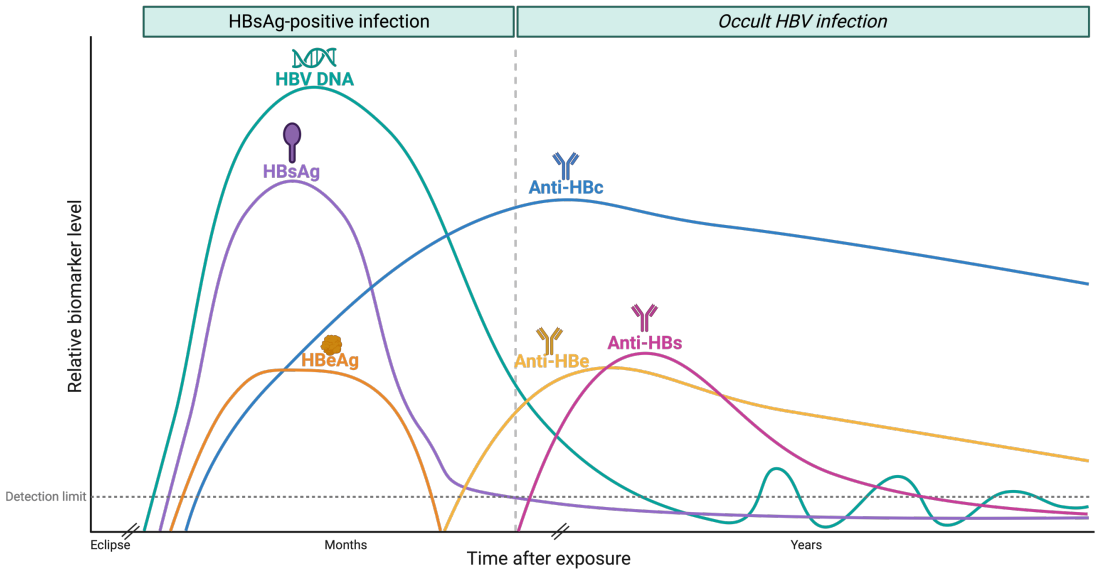


Figure 2[1]. Schematic depiction of relative levels of the virological blood biomarkers in the natural history of occult hepatitis B virus (HBV) infection. HBV DNA is the hallmark of replication. Hepatitis B surface antigen (HBsAg) constitutes the outer envelope component of HBV, where positivity for at least six months indicates chronicity. HBsAg is the first serological marker to appear, where seroconversion to HBV anti-surface antibodies (anti-HBs) occurs in occult HBV infection. Hepatitis B e antigen (HBeAg) is associated with viral replication. Antibodies against the core antigen (anti-HBc) are detectable throughout the natural history of infection, constituting the most sensitive marker of HBV exposure. Antibodies against the e antigen (anti-HBe) may be detectable independent of HBsAg or anti-HBs, but not without anti-HBc. The figure was devised and created by Michael Fu on Biorender.com.

Reactivation

Individuals with OBI carry a risk of reactivation of HBV infection. Both prospective and retrospective studies have demonstrated that HBV reactivation occurred in a pooled 6·5% of individuals with potential OBI (HBsAg-negative and anti-core antibody (anti-HBc)-positive) receiving immunosuppressive therapy[18]. During immunosuppression, immune control of HBV is lost, leading to the resumption of viral replication, often at high levels. This can lead to fulminant hepatitis[19], often observed clinically in patients who have reduced or ceased therapeutic immunosuppression, as a reconstituted immune system acts against HBV-infected hepatocytes. For this reason, the European Association for the Study of Liver Disease guidelines recommend prophylactic therapy in HBsAg-negative but anti-HBc-positive patients receiving those immunosuppressive regimens with relatively greater risk, including those who receive high-risk immunosuppressive therapies (e.g., rituximab) and solid organ and haematological transplant recipients[20]. It is hypothesised that there may be a higher risk of reactivation after previous chronic infection compared to after resolved acute infection, likely due to weaker or less durable immune control of residual viral reservoirs.

Liver cirrhosis and hepatocellular carcinoma

OBI may sustain a prolonged state of liver inflammation, as suggested by a prospective study in Japan; they showed the presence of cccDNA in all patients a decade after clinical recovery from acute self-limiting HBV infection, with liver fibrosis and mild inflammation detected in 89% of patients[21]. OBI seems to accelerate the progression of chronic liver disease and liver fibrosis in hepatitis C virus (HCV)

co-infection[22], where multiple studies have shown that the incidence and risk of HCC in HCV-infected patients with OBI were significantly greater than in HCV-infected patients without OBI[22–24]. However, one study in the USA found OBI in 10.7% of HBsAg-negative patients with advanced chronic HCV and HCC compared to 23.6% of similar patients but without HCC[25]. Data on other liver disease aetiologies is limited, with a recent study finding a 12.8% prevalence of OBI in obese patients undergoing bariatric surgery, suggesting OBI is a major risk factor for metabolic dysfunction-associated steatotic liver disease[26]. However, although OBI may be associated with intermittent or mild hepatic inflammation, serum liver enzyme levels (ALT/AST) are often normal and therefore lack sensitivity for identifying OBI.

HBV is a primary aetiological agent of HCC worldwide[27], with a well-established causal relationship between chronic HBsAg-positive infection and HCC. However, the association between OBI and HCC is less well-defined and has only been confirmed by retrospective studies, with no prospective studies. Retrospective studies found a high proportion (>60%) of Asian and European patients with cryptogenic HCC who had OBI[28–30]. In sub-Saharan Africa, where the HBV burden is high, a recent study found a high rate of anti-HBc positivity (16/36; 44%) and HBV DNA positivity (2/36; 6%, limit of detection 15 IU/mL) in HBsAg-negative patients with chronic liver disease and no identifiable cause[31]. Compared to HBsAg-positive chronic HBV infection, it is plausible that there is a lower oncogenic risk profile for individuals with OBI, considering the typically lower cccDNA levels and transcriptional activity, along with lower expression of viral proteins and necroinflammation that modulate hepatocarcinogenesis.

A prospective study in Hong Kong in HBV-infected patients who cleared HBsAg showed that 2·34% (7/298) developed HCC over a median follow-up of nine years, where all seven patients were over 50 years of age; HBsAg-negativity below the age of 50 was associated with a significantly lower risk of fibrosis and HCC[32]. Another Japanese prospective study with 82 cryptogenic cirrhotic patients showed that HCC incidence was 100% with and 17·6% without OBI at ten years (p=0.008; hazard ratio 8·25)[33]. Indeed, a body of literature suggests that OBI maintains the pro-oncogenic properties of HBsAg-positive HBV infection, with the ability to integrate HBV DNA into the host genome and persisting levels of pro-oncogenic X and pre-S/S viral proteins[34]. There have been reports of HBV integration in up to 75% of HCC cases with undetectable HBsAg levels[28,35], a comparable prevalence to that in HBsAg-positive HCC cases[36]. However, despite this evidence, whether OBI could mediate and accelerate liver damage remains a significant and debated topic, but an issue that should be carefully considered in patients with other factors of liver disease.

Transfusion-transmitted HBV infections

HBV transmission from individuals with OBI remains a concern in blood and transplant safety. Infectious HBsAg-negative but DNA-positive blood donations account for most of the residual risk of HBV transfusion-transmitted infections in developed countries[37], transmitting HBV to approximately 8-29% of recipients[38,39]. These transfusions may culminate in fatal complications, especially in recipients with existing immunological disturbances[40]. The prevalence of OBI (defined as HBsAg-negativity, HBV DNA-positivity) in blood donors worldwide mirrors patterns of HBV endemicity. OBI is detected at significant frequencies from 0.06% in low-endemicity countries to 12% in high-risk groups in high-endemicity countries[41]. The real-world prevalence may be even higher than estimated, as the current assays for HBV DNA utilised to detect OBI have suboptimal sensitivities[17]. The clinical relevance of OBI-associated transmission also varies by transfusion setting; in high-income countries like the UK, transfusions are predominantly given to immunocompromised recipients, whereas in low-to-middle-income countries, transfusions are more commonly given to pregnant women and severe childhood anaemia[42]. Immunocompromised recipients have a higher probability of infection with more severe outcomes after exposure, whereas transmission to pregnant women and neonates contributes to vertical and early-life infection with a much higher probability of chronic life-long infection; thus, implications of recipient profiles can have very different implications, even without considering screening sensitivity or residual risk frameworks.

HBV screening of blood donations in NHS Blood and Transplant (NHSBT), England, includes testing for HBsAg in individual donations and for HBV DNA by

nucleic acid testing (NAT) in pools of 24 donations[39]. Since the introduction of systematic national haemovigilance through the Serious Hazards of Transfusion (SHOT) scheme in 1996, only 16 confirmed cases of transfusion-transmitted HBV infection have been reported in the UK[248], highlighting the effectiveness of NHSBT donor selection, screening, and surveillance systems. This screening strategy had not, however, completely prevented transfusion-transmitted HBV infections from donors with OBI. 40 cases of possible transfusion-transmission of HBV were investigated by NHSBT between 2009 and 2018[39,43]. In 2018, a patient's death from acute HBV infection was traced back to a transfusion of a unit of red cells originating from a donation by a first-time donor, who met all the eligibility criteria for blood donation, but on further investigation, the implicated unit tested HBsAg negative, anti-HBc positive, HBV ID-NAT negative but HBV DNA was detected in a follow-up sample after concentration[44,45]. Another case of probable HBV transmission was linked to a red cell transfusion in 2015, where no other sources of infection were identified, but investigations showed one donor was anti-HBc positive but HBV DNA negative even after sample concentration[44]. It was estimated that at least 13 potentially infectious donations from donors with OBI would have remained undetected annually if the existing testing strategy of HBsAg and HBV DNA was continued, equating to a residual transmission risk of 3.1 per million donations[39]. While the estimated risk was very low in absolute terms, it still reflected a clinically relevant residual risk of HBV transmission that could have severe consequences for immunocompromised recipients of blood products from donors with OBI.

In blood donor screening, OBI donors are usually characterised by the presence of anti-HBc without detectable HBsAg and with very low viral loads (VLs; <200

IU/mL). Assuming HBV DNA is in plasma, a recent study revised the estimated minimum infectious dose of HBV DNA to 3 IU per transfusion[38]. With individual NAT screening, this required a PCR 95% limit of detection (LOD) of 0.150 IU/mL to avoid potential transmission[38]. Considering the typical 95% LOD of current NAT assays between 2 and 4 IU/mL, more sensitive assays are needed to avoid future transmissions from OBI donors unless anti-HBc assays are utilised to defer potentially infectious donations from the blood supply.

In response to likely HBV transmissions from OBI donors in England and elsewhere in the world[38,44], the UK Standing Advisory Committee on the Safety of Blood, Tissues, and Organs (SaBTO)[44] advised the Department of Health and Social Care that UK blood services should introduce routine anti-HBc screening to prevent further HBV transmission. Universal anti-HBc screening was introduced in May 2022.

Biomarkers to diagnose OBI

To diagnose HBV infections and OBI in NHSBT, three markers are utilised for universal screening, namely HBsAg, HBV DNA, and anti-HBc. Anti-HBs testing is further included in confirmatory testing.

HBV surface antigen

The undetectability or absence of HBsAg in OBI could be due to various factors. Low cccDNA levels in hepatocyte nuclei limit the expression of HBV transcripts and subsequent HBsAg expression, resulting in its undetectability[29]. Therefore, the first-line diagnosis of OBI highly depends on the sensitivity of the HBsAg assay. More sensitive assays may uncover greater frequencies of positivity and change a diagnosis from OBI to HBsAg-positive HBV infection. For example, while commercial HBsAg assays used in donor screening may have lower limits of detection (LLOD) of 50 IU/L[17], re-testing samples using assays with an LLOD of 5 IU/L detected HBsAg in often substantial proportions (1-48%) of samples that were HBsAg-negative in standard screening assays[46–48]. Development of an immune complex transfer chemiluminescence enzyme assay showed even greater sensitivity for HBsAg of 0.5 IU/L with high specificity, equivalent to sensitivities of current molecular assays[49]. However, these assays were developed to monitor HBV reactivation and have yet to be evaluated or validated for blood donor screening. Incorporating ultrasensitive HBsAg assays into diagnostic practice may detect minute levels of HBsAg in a substantial proportion of donors previously diagnosed with OBI. The presence of HBsAg may reflect the production of HBsAg from transcriptionally

active integrated HBV DNA, as previously described in chronic HBsAg-positive HBV infection[50]; their presence may indeed potentially contribute to the observed elevated risk of HCC in OBI[35]. Integration of HBV DNA into chromosomal DNA and subsequent rearrangement of genes may lead to a loss of HBsAg gene expression and reduced virion production. Processing defects in core antigen synthesis may block virus assembly and release from the cell, and lead to the accumulation of HBV DNA replicate intermediates in the liver[51]. Their adventitious integration may increase the risk of HCC development.

Other factors may hamper the expression of HBsAg. Mutations introduced during the error-prone reverse transcription of a pgRNA intermediate in HBV replication increase the likelihood of modulating HBsAg's expression, secretion, and synthesis[52]. Mutations in antigenic determinants in the surface gene may result in an escape of detection of HBsAg by currently available assays. The most common mutants reside in the exposed major hydrophilic region on the S protein, where the 'a' determinant is found[53]. Mutations in the 'a' determinant alter the disulphide bonds in cysteine residues, preventing immune recognition of HBsAg by specific anti-HBs antibodies[53] and resulting in the escape of detection by assays using monoclonal tracers[54]. Another issue is the failure to detect immune-escape mutations of HBsAg in individuals with HBV reactivation in the presence of anti-HBs from past HBV exposure[55] or vaccination[56]. The overlapping polymerase and surface ORFs also enable drug-selected mutations in the reverse transcriptase/polymerase to hinder HBsAg detectability[57]. These challenges necessitate highly sensitive assays utilising anti-HBs probes targeting multiple epitopes of HBsAg to detect these rarer S variants consistently. Assays that use multivalent polyclonal tracer antibodies that bind a range

of mutated HBsAg's have been developed[58], but the sensitivities of these assays remain variable[54].

Although OBI is defined as HBV DNA positivity in the absence of detectable HBsAg, failure to detect HBsAg because of escape mutations represents a very different infection outcome from typical OBI, where HBsAg non-detection is the result of suppressed expression of antigenically normal HBsAg[59]. HBsAg in the former may be present in high titres comparable to those of HBsAg-detectable infections and potentially with higher HBV DNA VLs[17], conferring high transmissibility by transfusion and potentially other routes.

Non-detection of HBsAg may also result from intracellular retention of viral proteins, leading to low-level or absent extracellular HBsAg in the bloodstream. Mechanistically, rare mutations in the small S envelope protein were found to prevent HBsAg secretion, resulting in accumulation in the endoplasmic reticulum/Golgi apparatus[60,61]. Deletions in the S-promoter region also reduced transcript levels of middle and small S proteins, where subsequent overexpression of large S protein led to intracellular retention of non-secretable HBsAg[62].

Finally, excess anti-HBs antibodies in the presence of anti-HBs/HBsAg immune complexes may mask the detection of HBsAg[63] since HBsAg assays typically can only bind non-immune complexed protein. Immune complexed HBsAg has been detected in patients with hepatocellular carcinoma previously diagnosed with OBI[64] and in acute and chronic HBV infections[65], where non-detection was not the result of mutants in major HBsAg epitopes[66]. Indeed, a subset of OBI patients may have

detectable HBsAg in the peripheral blood using assays that could pre-dissociate HBsAg from immune complexes[67].

HBV DNA

In line with the recent consensus definition of OBI[17], the optimal diagnostic test for HBV DNA is the analysis of extracted DNA from liver tissues using highly sensitive techniques[59]. HBV DNA is only intermittently detectable in the blood[38,68,69], usually with concentrations of less than 200 IU/mL[38,70,71]. However, liver biopsy is rarely available and unfeasible for most individuals due to its invasive nature, especially in generally healthy blood donors, and the small biopsy volumes may limit HBV detection due to irregular distribution of HBV DNA in the liver[72]. Serum or plasma analysis is currently the most utilised sample to identify OBI cases.

OBI diagnoses depend not only on HBsAg but also on HBV DNA assay sensitivities. When mini-pools are used for NAT (MP-NAT) testing of blood donors, those with OBI are often not detected since HBV DNA levels are usually lower than the LOD for pooled samples in current assays[73]. Using a prospective repository of donor-recipient pairs in Italy, 50% of DNA-positive samples detected by individual donation NAT (ID-NAT; LOD 3.7 IU/mL) were not identified by 6-MP-NAT (LOD 22.2 IU/mL)[71]. As a result, two cases of HBV transmission were confirmed by donor-recipient sequence identity. One recipient received a red blood cell (RBC) unit from an OBI donor with a viral load <12 IU/mL and developed acute liver failure 22 weeks post-transfusion. The second recipient was on long-term immunosuppressive treatment and experienced acute HBV infection with life-limiting liver failure[71]. There

may have been more transmissions than found in this study due to the lack of samples from several donor-recipient pairs for ID-NAT testing. This study emphasises the need for increased ID-NAT sensitivity compared to MP-NAT to detect OBI in donors, to allow sequence analysis of donor-recipient viruses and to prevent HBV transmission to recipients.

However, many OBI donations remain undetected, even with ID-NAT[73]. NHSBT lookback identified transmission of HBV from a donor with undetectable DNA (ID-NAT LOD: 8 IU/mL)[74]. With a 95% LOD of 3.4 IU/mL, mathematical modelling suggested that 3.3% and 14% of undetectable OBI donations may result in transmission with 20 mL and 200 mL of plasma, respectively[75]. This modelling was corroborated by a recent study of 24 anti-HBs negative recipients of components from three HBsAg-negative and DNA-negative at screening (LOD 3.4 IU/mL) repeat donors in Slovenia that, after centrifugation with increased volumes of plasma, managed to confirm nine recipients newly HBV-infected with >99% donor-recipient sequence homology[38]. 37.5% of susceptible recipients being transmitted with HBV from OBI donors highlights an urgent need to develop more sensitive HBV DNA PCRs to exclude infectious donations with low viral loads. This study revised the estimated minimal infectious dose to 3 IU of HBV DNA in 20 mL of plasma, requiring an estimated LOD of 0.15 IU/mL[38]. Given that 1 IU corresponds to approximately 5 copies of HBV DNA, a LOD of 0.15 IU/mL would require reliable detection of fewer than one viral copy per millilitre. Achieving this level of sensitivity is challenging, considering current NAT assays have 95% LODs ranging from 4 to 12 IU/mL[76]. Moreover, at such low viral loads, stochastic sampling effects become relevant,

meaning that failure to detect HBV DNA in a tested aliquot may still be compatible with a non-zero viral burden within the transfused unit.

As supported by findings of absent HBV DNA in archived samples of infectious OBI donors by transfusion[38], OBI donors are often characterised by intermittent viraemia, where periodic testing of HBV DNA is necessitated[69,77]. Such testing may be impractical, especially in many low-to-middle-income countries (LMICs) where HBV is highly endemic, and the cost of performing ID-NAT is already economically prohibitive. When ultrasensitive HBV DNA testing is not feasible in low-endemic countries, anti-HBc is currently advocated as a surrogate marker to identify potentially infectious donations with fluctuating viraemia and improve blood safety[59], since almost all OBI donors are anti-HBc positive[70].

Considering the very low VLs in OBI compared to the detection limits of current commercial assays, it is plausible that a larger proportion of anti-HBc-positive donations containing low levels of HBV DNA remain currently unidentified.

Antibodies against the HBV core antigen

Anti-HBc antibodies usually persist during HBV infection and remain detectable after recovery[78]. Anti-HBc can be the only detectable HBV serological marker in OBI blood donors[79,80]. What proportion of healthy individuals, such as blood donors, who test anti-HBc positive but NAT-negative in blood, harbour replication-competent cccDNA in the liver is currently unknown. Similarly, it is not well-

understood what factors trigger replication beyond immunosuppression[81]. Without liver biopsies to ascertain cccDNA presence, anti-HBc screening may be necessary to exclude potentially infectious donations.

Whilst anti-HBc assays are more economical and practically more straightforward to implement than ID-NAT testing, the high false-positive rate of current assays results in the unsustainable deferral of otherwise healthy donors[82], particularly donors with rarer blood types[83]. Some blood establishments retest reactive samples with an alternative assay to discriminate between true and false positives. However, many of these assays are still not fully discriminatory; hence, developing a highly specific confirmatory test would help reduce this diagnostic uncertainty. Furthermore, anti-HBc testing becomes a particular issue in HBV-endemic countries where the prevalence of anti-HBc can approach 50% and only a tiny proportion of anti-HBc-positive donations have detectable HBV DNA[82] using currently available assays, which may underestimate the true prevalence of HBV DNA in anti-HBc positive and HBsAg-negative donations.

Anti-HBc screening does not identify all OBI donations; extreme NAT sensitivity is also required to eliminate DNA-containing donations[84]. For example, the absence of anti-HBc does not rule out seronegative OBI. Estimated to comprise between 1 and 20% of all OBI individuals[17], this status may result from the progressive disappearance of antibodies following the resolution of acute HBV infection or from a lack of circulating antibodies from the beginning of HBV infection, as shown in the woodchuck *hepadnavirus* model[85]. However, the prevalence of seronegative OBI in many countries is unknown, requiring future work to study the clinical significance of

seronegative OBI, its risk for TTI, and the utilisation of non-serological markers to detect these cases.

Antibodies against the HBV surface antigen

Accumulating evidence suggests that the infectivity of blood transfusions from OBI donors is significantly reduced and potentially neutralised when anti-HBs antibodies are present compared to donors with isolated anti-HBc status[38,40,86–90]. Indeed, studies found HBV DNA detection highest in anti-HBc positive and anti-HBs negative blood donors[2,70], where anti-HBs in the bloodstream may neutralise infectious HBV particles[82]. A European study found all recipients of HBsAg-negative, anti-HBs-negative, and HBV DNA positive fresh frozen plasma (FFP) were infected, whilst none of the three recipients of HBsAg-negative, anti-HBs positive, and HBV DNA negative FFP had markers of infection[40]. Similarly, no transmission was reported in 22 recipients of anti-HBs positive blood components, whereas 10 of 37 recipients of anti-HBs negative components were HBV infected[88]. A follow-up study of a donor with intermittently low DNA levels <10 IU/mL and anti-HBs >2000 IU/L found no posttransfusion HBV from RBC or FFP components over seven years[91]. In chimeric mice, transfusion of anti-HBs negative donor blood confirmed HBV transmission with detectable cccDNA and blood HBV DNA[92]. Conversely, the other transfused anti-HBs positive blood (34 IU/L) did not transmit HBV[92]. Therefore, identifying anti-HBs negative but anti-HBc positive donors is crucial in preventing transfusion-transmitted infections from donors with OBI. These transfusions could lead to acute fulminant

HBV with fatal outcomes in immunosuppressed recipients and recipients with sepsis, as shown by recipient-induced investigations[89].

However, anti-HBc screening does not identify isolated anti-HBs OBI, hypothesised to occur from long-term persistence of OBI from vertical transmission or from vaccine breakthrough infection[93] that may have extremely low viral loads below NAT LODs[94]. It has been shown that 5.65% of HBsAg-negative, anti-HBc negative but anti-HBs positive vaccinated children and young adults recruited across Taiwan for an epidemiological study of vaccine-preventable diseases had detectable HBV DNA (26/460)[95], suggesting potential vertical or horizontal transmission before infant vaccination was administered. Long-term loss of anti-HBc may occur before anti-HBs loss after acute HBV infection, with up to 11% of OBI donors found to be DNA-positive[76,96]. There has been a recent report of HBV transfusion-transmitted infection from an isolated weakly anti-HBs positive donor (10.7-95.8 IU/L) with transient detectable levels of HBV DNA (LOD: 3.1 IU/mL) to a recipient not immunocompromised that was HBV-negative pre-transfusion but developed typical HBV infection after transfusion with confirmed sequence identity[94]. Although the vaccination status of the donor was unknown, this novel case highlights that potentially infectious isolated anti-HBs OBI donors may be missed by screening strategies currently utilised by blood establishments. Alternative biomarkers and screening strategies are needed to identify these cases.

Although anti-HBs presence may significantly reduce the likelihood of transmission by transfusion[40], the protective level of anti-HBs is not well-defined, and anti-HBs presence does not invariably preclude infectivity. Neutralising anti-HBs

prevents the intracellular spread of HBV but does not block the replication of the intracellular virus[81]. Indeed, several cases of transmission with anti-HBs positive donations (<50 IU/L) have been described. Recipient-triggered lookback in Australia found a pretransfusion serology-negative recipient who developed acute HBV infection post-transfusion, identifying one donor who was anti-HBc positive and NAT-negative (LOD 10.4 IU/mL) with an anti-HBs titre of 36 IU/L[97]. Although sequence homology could not be assessed to confirm transfusion-transmitted infection, this case highlights that low anti-HBs levels may not prevent transmission and that NAT assays must be more sensitive to detect the low viral loads characterising OBI.

The infectivity of anti-HBs containing blood components for immunodeficient recipients has yet to be systematically investigated; caution should be taken when these components are transfused to immunodeficient/unimmunised recipients[98], especially since transfusion of around 50% of blood components in Western Europe is to patients with some degree of immunodeficiency[9]. However, one study found that an anti-HBs positive (12 IU/L) OBI donor infected two immunocompetent recipients with 180 IU/mL of HBV DNA[99], suggesting higher viral loads in OBI may overcome the relatively weak neutralising capability of low anti-HBs levels and transmit HBV, even in the case of immunocompetent recipients. Thus, the evidence provided in this section shows that low anti-HBs levels may not be protective from infectivity when HBV DNA is present. Donors with anti-HBs >100 IU/L, incorporating a precautionary safety margin[97], could be considered eligible for donation since no transfusion-transmitted infection has been reported from donors with high anti-HBs levels.

Scope of thesis

Given the recent introduction of anti-HBc screening in NHSBT, investigations into the detection of blood donations positive for OBI seem timely, as does the investigation into the effectiveness of the screening strategy for anti-HBc. This thesis entails four aims:

1) Improving the sensitivity of HBV DNA detection and thus the detection of further anti-HBc positive donations that contain HBV DNA (OBI), allowing for potential stratification of infectious vs. non-infectious blood donors.
2) Investigating the effectiveness of alternative methods for detecting HBV DNA, other than real-time PCR, to obtain more detailed genetic information on HBV and other pathogens in blood donations.
3) Investigating the effectiveness of the current anti-HBc screening strategy of identifying true-positive donations and the effects of screening on the blood supply.
4) Investigating the screening and deferral strategies for blood donations from individuals infected with or at risk of OBI across different blood establishments globally, providing insight into the utility of screening in preventing transfusion-transmitted infections.

Statement of Candidate Contribution

This section describes the candidate's contributions to the experimental work and data analysis presented in this thesis. NHSBT performed all sample collection, routine diagnostic testing, and donor population data collection.

For Chapter 1, all experimental work was performed by the candidate, except for the ultracentrifugation method, which was performed by the Scottish National Blood Transfusion Service, and next-generation sequencing, which was performed by colleagues at the Peter Medawar Building. The candidate performed all data analysis for this chapter, except for the primary bioinformatic processing of the next-generation sequencing data.

For Chapter 2, the candidate prepared all samples, including nucleic acid extraction where required, which were then sequenced at the respective laboratories. Each laboratory then performed the initial bioinformatic processing of its sequencing data to generate per-base genome counts; the candidate conducted all subsequent downstream analyses.

For Chapter 3, the candidate performed all experimental work beyond the routine NHSBT testing and conducted all data analyses. For Chapter 4, the candidate performed all data collection and analysis. The candidate was responsible for data interpretation and drafting of the entire thesis.

Chapter 1: Increasing sensitivity for HBV DNA detection and risk stratification for occult hepatitis B virus infection

Most of this chapter is based on the published papers:

Fu MX, Simmonds P, Andreani J, et al. Ultrasensitive PCR system for HBV DNA detection: Risk stratification for occult hepatitis B virus infection in English blood donors. J Med Virol. 2023 Oct;95(10):e29144. doi: 10.1002/jmv.29144.

Fu MX, Larralde O, Mayne R, et al. Use of polyethylene glycol precipitation and ultracentrifugation to enhance the sensitivity of hepatitis B virus DNA detection. J Clin Virol. 2025 Jun;178:105802. doi: 10.1016/j.jcv.2025.105802.

Introduction

At NHSBT, all donations were screened for HBsAg (PRISM, Abbott Diagnostics, LOD 0.08-0.10 ng/mL) and HBV DNA in pools of 24 (Roche Cobas MPX; 95% LOD 1.4 IU/mL; calculated 95% LOD 33.6 IU/mL in individual donor level when tested in pools of 24) followed by individual NAT if the pool was positive. If donations were anti-HBc-positive on screening (Architect anti-HBc II, Abbott Diagnostics), testing was repeated in duplicate and considered repeat reactive if two of three tests were positive. Anti-HBs screening followed (Architect, Abbott Diagnostics), and if levels were below 100 IU/L, the donation was sent to NHSBT Microbiology Services Laboratory for confirmatory anti-HBc testing (Murex anti-HBc, DiaSorin), antibodies to HBeAg (anti-HBe; Vidas), and individual NAT (Procleix Ultrio Elite dHBV Assay, Grifols; 95% LOD 4.5 IU/mL). HBV DNA-positive donations were sent for genotyping and measurement of VL by the Blood Borne Virus Unit, Blood Safety, Hepatitis, STI & HIV Division, UK Health Security Agency (UKHSA). All anti-HBc confirmed positive donors were permanently deferred from

blood donation. All donations with any HBV reactivity, whether confirmed or not, were discarded.

The introduction of anti-HBc screening at NHSBT has resulted in the deferral of donors screening positive, thereby eliminating the risk of transfusion-transmitted infections from donors with OBI who present with HBsAg negativity, anti-HBc positivity, and HBV DNA positivity. The minimum infectious dose of HBV DNA is 3 IU/mL, requiring assays with a LOD of 0.150 IU/mL to avoid potential transmission via blood components containing 20 mL of plasma[38]. However, the LOD of commercially available ID-NAT assays, such as those used at NHSBT after testing for anti-HBc, is much higher, between 2 and 4 IU/mL. Therefore, the development of a more sensitive assay could detect a greater proportion of HBV DNA positivity from anti-HBc positive donations. The sensitivity of the detection could be improved by concentrating HBV particles from larger volumes of plasma, where high-speed ultracentrifugation was reported to pellet HBV from >10 mL of plasma[100]. However, the availability and technical difficulties of this ultracentrifugation method present major limitations to its wide-scale use in clinical virology laboratories.

Polyethylene glycol (PEG) has long been used to precipitate and concentrate HBV particles for in vitro infection studies[101]. PEG has been successfully used to enhance viral nucleic acid recovery from environmental samples[102], such as in monitoring SARS-CoV-2 circulation[103] and generating viral metagenome sequence libraries[104]. The use of PEG for viral nucleic acid concentration in human plasma has been studied less and lacks evidence in clinical and transfusion contexts. Previous research with HBV has suggested that PEG was marginally less effective than

ultracentrifugation for concentrating HBV DNA from plasma[105,106]. However, VLs were not quantified in these studies, and the input plasma volumes for PEG (maximum 6 mL) and ultracentrifugation (maximum 12 mL) protocols were relatively low.

Further, the introduction of anti-HBc screening has resulted in a large number of donations being deferred from the blood supply, with 0.23% of all donations in 2023 testing confirmed anti-HBc positive[107] and would be deferred from donation. This is especially important as anti-HBc positive donors mostly come from HBV-endemic areas[107], who may be of rarer blood groups. Since not all, and most likely a very small proportion of, anti-HBc positive donors have circulating, infectious HBV DNA in their blood, many donations may indeed be safe for transfusion (in the absence of other transfusion-transmitted infections), and additional markers may be utilised to more accurately stratify potentially infectious donations, enabling the retention of safe blood units for transfusion.

The aim of this chapter was to develop an ultrasensitive PCR system for HBV DNA detection through investigations of the clinical performance of various extraction and amplification methods. HBsAg-negative, anti-HBc-positive blood donations with low anti-HBs levels (<100 IU/L), more likely to be associated with infectivity due to low levels or absence of neutralising antibodies[38], were subsequently re-tested for HBV DNA using the most sensitive assay. Risk factors in these blood donors that were potentially predictive of detectable DNA were also investigated.

Materials and methods

Samples and controls

The 4th WHO International Standard for HBV DNA (National Institute for Biological Standards and Control, ref 10/266) was used to calibrate the PCR assay in IU/mL. Following manufacturers' protocols, it was reconstituted and extracted with the Roche Large Volume Extraction Kit (Roche, Basel). For assay calibrations, serial dilutions of the standard were made in a buffer solution containing 1 mM sodium citrate at pH 6.4, 50 µg/mL of herring sperm carrier DNA and 0.14 U/µL RNAsin (Thermofisher Scientific).

A control panel of 26 known HBV-positive plasma samples were obtained from either HBsAg-positive blood donors (n=22) or OBI donors (anti-HBc and HBV DNA-positives without HBsAg; n=4) from NHSBT routine screening (Table 1) and utilised for PCR system development. To determine the frequency of HBV DNA positivity in anti-HBc-positive donors, the first available 195 plasma packs that tested anti-HBc-positive, DNA-negative by individual NAT and anti-HBs <100 IU/L between 30/May/2022 and 31/Dec/2022 were received from NHSBT.

Table 1[2]. Characteristics of the sample panel: HBsAg status, genotypes assigned by the current study, viral load found by this study using the genotype and viral loads reported from NHS Blood and Transplant (NHSBT) Microbiology Services Laboratory and the UK Health and Security Agency (UKHSA). Samples tested DNA-positive on initial NHSBT qualitative screening assay, and a separate in-house quantitative assay was used at NHSBT to re-test the samples for HBV DNA viral loads; differences in testing aliquot/time and assay sensitivity could have affected the detection of HBV DNA using the in-house quantitative assay. *The three selected samples for PCR assay development, where Sample 13 was further utilised as an internal control calibrated against the HBV DNA 4th International Standard; $^{+}$Genotyping at UKHSA not done for six samples as viral load was undetectable or below the level of quantification; $^{\#}$Genotyping at UKHSA was not done for five samples due to the COVID-19 pandemic. OBI: occult hepatitis B virus infection.

Sample	*HBsAg status*	*Genotype assigned by this study*	*Genotype from UKHSA*	*Viral Load (IU/mL) found by this study*	*Viral Load (IU/mL) from NHSBT in-house assay*
Sample 1	+	D2	D2	533	919
Sample 2	+	D	Not done[+]	3	Not detected
Sample 3	+	A	A2	2715	2960
Sample 4 (OBI)	-	A3	Not done[+]	3	Not detected
Sample 5	+	B4	B4	55	56.9
Sample 6	+	E	E	42	240
Sample 7	+	A	Not done[+]	25	Not detected
Sample 8	+	D1	D1	228	482
Sample 9*	+	E	Not done[#]	1871	3520
Sample 10	+	D3	D	22	18.7
Sample 11	+	A2	A2	7115	7980
Sample 12	+	C2	C2	670	947
Sample 13*	+	A2	Not done[#]	475,689,329	102,000,000
Sample 14	+	A	A	599	712
Sample 15	+	E	E	141	379
Sample 16 (OBI)	-	A	Not done[+]	25	5.07
Sample 17	+	D3	D3	619	661
Sample 18	+	B	B	4,873,759	18,800
Sample 19 (OBI)	-	C5	Not done[+]	27	49.9
Sample 20	+	C5	Not done[#]	48,242,946	1,720,000
Sample 21	+	C5	Not done[#]	41	87.5
Sample 22*	+	D1	D1	3881	4390
Sample 23	+	A	A2	53	190
Sample 24	+	A1	Not done[+]	38	99.9
Sample 25	+	D	Not done[#]	550	2860
Sample 26 (OBI)	-	A	Not done[+]	24	13.9

Ethics statement

Signed consent was obtained from each donor at the time of donation. Donor consent to NHSBT includes holding information about their health, attendance, and donations. It also covers research which improves our knowledge of the donor population. This study was approved by the Blood Supply Clinical Audit, Risk and Effectiveness Committee of NHSBT on 19th April 2022 and 8th January 2023.

Testing data

Anonymised virological data (HBV DNA VL, genotype, anti-HBs titre and anti-HBc sample to cut-off [S/CO] ratio) were obtained for 40 OBI donors identified from the introduction of HBV DNA screening in 2009 until 2022, and for the first 134 of 195 donations assayed in the current study. These were obtained from NHSBT.

DNA concentration methods

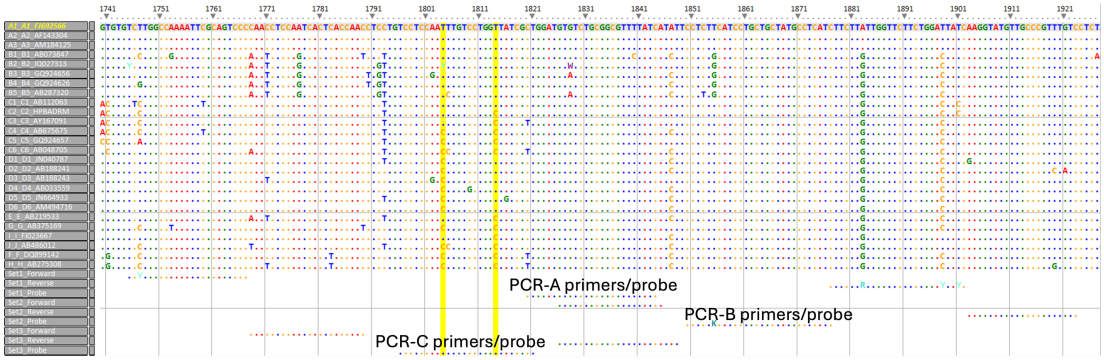
Seven extraction methods were compared. Manufacturer instructions were followed for the Zymo Quick-DNA/RNA Viral Kit (Zymo Research; Zymo), Zymo Quick-DNA/RNA Viral 96 Kit (Zymo Research; Zymo96), DNeasy Blood and Tissue Kit (Qiagen; DNeasy), MagMAX Kit (Thermofisher Scientific; Kingfisher), and Roche High Pure Viral Nucleic Acid Large Volume Kit using 2500 µL extraction volume (Roche2.5). To attempt to increase the amount of extracted nucleic acid, modifications were made to the QIAamp DNA Blood Mini Kit (Qiagen; QIAamp) protocol: 1 µL buffer solution (described in 'Samples and controls') was added to 200 µL Buffer AL, ethanol was increased from 200 µL to 230 µL and 60 µL buffer AE was used instead of 50 µL. Moreover, novel modifications to the Roche2.5 protocol to

utilise larger extraction volumes[108] (Roche5): 5000 µL instead of 2500 µL of plasma was added to 2.5 mL instead of 1 mL of binding buffer, with overnight lysis at 37°C (replacing lysis at 75°C for 20 minutes), and addition of 2 mL instead of 1 mL of the binding buffer after lysis. All extraction methods were performed manually except the MagMAX kit, which was processed using a Kingfisher Apex Benchtop Sample Prep. Comparisons of the HBV VL across the extraction methods for each sample investigated each method's sensitivity and compared the recovery of HBV DNA between methods.

Real-time PCR methods

Three published PCR methods for HBV DNA amplification were selected for comparison[109–111] (PCR-A to C; Table 2). The potential effect of HBV genetic variability on the effectiveness of HBV DNA amplification in the three assays was investigated by aligning sequences of their primers and probes with prototype sequences of each HBV subgenotype (Figure 3). Subsequently, the sensitivity of these three PCR methods was assessed by testing serial dilutions of three samples selected from the panel and standardised against the International Standard.

Figure 3[2]. Alignment of primer/probe sets with reference sequences of HBV subgenotypes. The two base mismatches in the PCR-C probe are highlighted in yellow.



For comparison of PCR and extraction methods, 2.5 µL of extracted DNA was amplified in a total 20 µL reaction volume using the Quantitect Probe RT-PCR kit (Qiagen) on an Applied Bioscience StepOnePlus Thermocycler. Following a comparison of different annealing temperatures, the final protocol was used for all measurements: polymerase activation of 15 minutes at 95°C followed by fifty cycles of denaturation at 95°C for 15 s and a combined annealing and extension phase at 60°C for 60 s. Final concentrations of 1 µM for each primer and 0.5 µM for the probe were used. All samples and controls were measured in duplicate. Sample 13 was utilised as a secondary standard as an internal run control to quantify HBV and account for intra-assay variation. This was calibrated against the 4th HBV DNA International Standard (National Institute for Biological Standards and Controls, ref 10/266), and serial dilutions were included in each assay to convert Ct values to IU.

The sensitivities of PCR methods were evaluated, and the best method was used for testing anti-HBc-positive donations. Multiple replicates of sequential 1:2 dilutions of the International Standard from 45.36 IU to 0.09 IU of HBV DNA were measured to investigate the analytical sensitivity of the optimal HBV DNA extraction

and PCR assay. The anti-HBc-positive donations were assayed once, but controls were tested in duplicate. Initial reactive donations were re-extracted and re-assayed twice, with a positive donation defined as DNA-positive in at least one repeat.

Nested PCR and sequencing

HBV genotypes in all DNA-positive samples were identified by phylogenetic analysis of sequences amplified by previously described primers S2 and S3 targeting a continuous sequence in the S gene (Table 2)[112]. The following conditions were used: initial denaturation at 95°C for 2 minutes, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 minute. A final extension at 72°C for 5 minutes was then followed by a 4°C hold. 10 μL of extracted DNA was added to 40 μL of PCR mix in the first round, with 2 μL of the product added to 48 μL of PCR mix in the second round. PCR mix constituted 5X Colorless GoTaq Reaction Buffer (10 μL; Promega), GoTaq G2 DNA Polymerase (0.5 μL; Promega), 0.2 μL each primer, GeneAmp dNTP Blend (1 μL; Life Technologies), and DNA-RNA free water up to the required volume.

Table 2[2]. Sequences of the nested PCR primers utilised for sequencing.

Primer set	*Forward primer*	*Reverse primer*
S2 (outer)	CATCAGGAYTCCTAGGACCCCT	GAGGCATAGCAGCAGGATGMAGAGG
S2 (inner)	CGTGTTACAGGCGGKGTKTTTCTTGT	ATGATAAAACGCCGCAGACACATC
S3 (outer)	GACTTCTCTCAATTTTCTAGGGG	AGTAAACTGAGCCAAGAGAAACGG
S3 (inner)	GATGTGTCTGCGGCGTTTTATCAT	ACGGACTGAGGCCCACTCCCATAG

Positive samples identified by gel electrophoresis were sent for Sanger sequencing to Source Bioscience (Cambridge, UK). Sequences were aligned using SSE[113] to a 90% consensus sequence of all HBV subgenotypes. Phylogenetic trees were constructed by maximum likelihood in MEGA11[114], with 100 bootstraps resampling to determine the robustness of groupings. Trees included reference sequences of all HBV subgenotypes.

Further method development

Whilst the previously described experiments in this chapter were performed in the years 2022-2023, the development of further methods was performed afterwards in 2024-2025. Further methods were developed following the application of the best method, since a larger extraction volume was found to be associated with a greater sensitivity. These additional methods were compared to the Roche5 method (Method 2) and a method using a more standard extraction volume of 0.2 mL of plasma, which was shown to be sensitive compared to other extraction methods using similar input volumes of plasma (QIAamp; Method 1). The further methods were:

Method 3: using the manufacturer-recommended plasma input volume for PEG concentration, this method involved pre-extraction concentration of HBV from 10 mL of plasma following the manufacturer's instructions for the PEG Virus Precipitation Kit (Abcam, ab102538). The pellet was resuspended in 200 µL of Virus Resuspension Solution and 800 µL of phosphate-buffered saline (PBS) and extracted the whole volume with the Roche High Pure Viral Nucleic Acid Large Volume Kit, following manufacturer instructions.

Method 4: aiming to improve sensitivity from Method 3 by increasing plasma input volume past the manufacturer's protocol, this method involved modifications to the 10 mL protocol by doubling the input volume of plasma (20 mL) and 5X PEG solution (5 mL) and resuspending the pellet in 400 µL of Virus Resuspension Solution and 2.1 mL of PBS, then extracting the whole volume with the same kit.

Each sample was concentrated and extracted in the same runs for Methods 2-4, including two HBV negative controls to confirm specificity.

Method 5: as a comparator to PEG for effective HBV DNA concentration, HBV was pelleted by ultracentrifugation of 35 mL of plasma at 30,000 rpm for 3 hours at 15°C using a rotor SW32 Ti of Optima XPN-100 ultracentrifuge (Beckman Coulter). Pellets were resuspended in 1 mL of Working Solution of the Roche High Pure Viral Nucleic Acid Large Volume Kit, and the whole volume was extracted following the manufacturer's instructions. Method 5 was performed by the Scottish National Blood Transfusion Service, since the laboratory containing the ultracentrifuge machine at the Peter Medawar Building was closed during this period of work. Only the 12 lowest VL samples were assayed through Method 5 to minimise any potential risk of contamination.

Feasibility experiments were conducted to assess the relative effectiveness of PEG concentration. Firstly, 200 μL aliquots of plasma containing 96,000 IU of HBV DNA were added, where necessary, to excess plasma that tested negative for screened blood-borne viruses to make up 200 μL, 5 mL, 10 mL, and 20 mL of plasma. These were extracted using the methods listed above. Method 5 was not evaluated in these feasibility experiments, as it was an established method to concentrate DNA from large volumes effectively. Since these experiments were conducted in the final year of the DPhil, there was a lack of time to then test the anti-HBc positive plasma packs using the newly developed methods.

Next-generation sequencing

Targeted enrichment next-generation sequencing (NGS) was performed to investigate the relative performance of PEG concentration from 20 mL of plasma to standard extraction from 5 mL of plasma (Methods 4/3 and 2, respectively). cDNA synthesis and library preparation were performed according to a previously described protocol[115], with a modification using a mixture of 2.5 μL of cDNA and 2.5 μL of original DNA extract (gDNA) as input template. Extracts for each sample were assayed in the same run with appropriate controls. Samples were sequenced using an Illumina NovaSeq 6000 (paired 150 bp) and were analysed via Castanet[115] to obtain deduplicated reads, viral genome coverage, read depth statistics, and consensus HBV genomes.

Statistical analyses

Based on linear regression, average Ct values of serial dilutions of positive controls were converted to IU. Linear regression determined R^2 for serial dilutions of PCR methods. Data normality was assessed with the Shapiro-Wilks test. All non-normal data are reported as median [interquartile range]. Ct values were compared via the Friedman test with post-hoc Dunn's multiple comparison testing. Spearman's test correlated HBV VLs of the control panel tested by our ultrasensitive system versus clinical testing. Fisher's exact tests compared categorical variables for risk stratification, while Mann-Whitney U tests compared continuous variables. Where shown, confidence intervals were calculated as the 95% binomial confidence intervals. Receiver operating characteristic (ROC) analysis investigated the sensitivities and specificities of HBV serological markers that may predict DNA positivity. The Chi-squared test compared the samples detected, and non-linear log-log regression

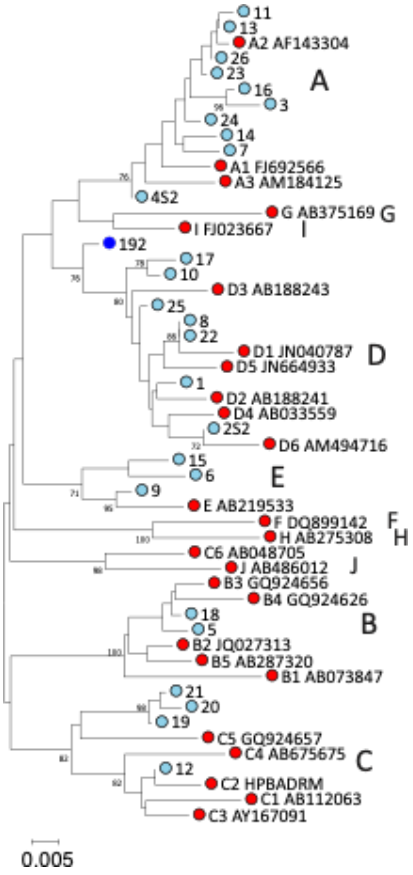
compared expected and calculated VLs. All analyses were performed with GraphPad Prism (v9.5.1 and v10.4.0, LLC), except Probit analysis on SPSS (v28.0.0.0, IBM). Statistical significance was set at $p \leq 0.05$.

Results

Characterisation of HBV plasma panel

26 plasma samples were utilised to assess the sensitivity and specificity of a range of PCR assays for HBV DNA (Table 1). To confirm genotype assignments and further characterise the HBV strains in the control samples genetically, part of the S gene was amplified by nested PCR and sequencing of the second-round amplicon. All 26 samples yielded amplified products which could be assigned as genotypes A-E by phylogenetic comparison (Figure 4). These assigned genotypes matched the available genotypes determined by the UKHSA (Table 1).

Figure 4[2]. Phylogenetic tree of the sample panel (coloured in cyan) and the sequenced DNA-positive blood donation found in this study (Donation 192; coloured in navy blue), with reference sequences of HBV subgenotypes (coloured in red). Sample 2 and Sample 4 could only be sequenced by S2 primers and not S3 primers, thus are named 2S2 and 4S2.



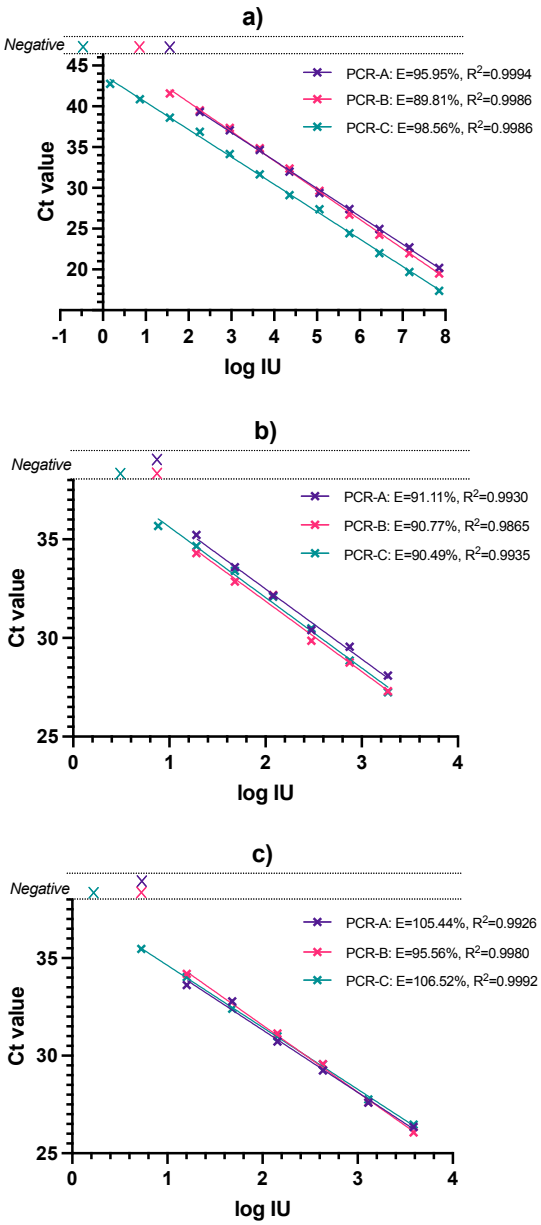
Comparison of PCR methods

First, we investigated the potential effect of HBV genetic variability on the effectiveness of HBV DNA amplification in the three selected PCR assays (Table 3). Primers and probes from PCR-A and PCR-B showed 100% identity with all subgenotypes, but the PCR-C probe mismatched several HBV variants (including genotypes D1 and E) at two base positions, potentially influencing target hybridisation affinity (Figure 4). To investigate whether this influenced assay sensitivity, three samples (sample 13, genotype A2; sample 9, genotype E and sample 22, genotype D1) were selected for further evaluation. Ct values obtained from the dilution series of Sample 9 (genotype E) and Sample 22 (genotype D1) that showed mismatches to the PCR-C probe sequence were compared with those of Sample 13 (genotype A2 – no mismatches) in the three assays (Figure 5).

Table 3.[2] Sequences of the three primer/probe sets, details about the source of sequences and reported sensitivities. Modifications made to PCR-A are detailed in the table.

Primer/probe set	*Sense primer*	*Antisense primer*	*Probe*	*Source and Modifications*
PCR-A	TCYTGGCCAAA ATTCGCAGTCCC	GRTARTCCAGAA GAACCAAYAAGA AG	FAM-CTGGATGTGT CTGCGGCGTTTTATC -BHQ1	Previous 50% LOD 8.4 IU/mL using International Standard (genotype A2)[111]. The probe position was altered to change non-matching ends, and the antisense primer was modified to a 90% consensus sequence of all HBV subgenotypes.
PCR-B	GTGTCTGCGGC GTTTTATCA	GACAAACGGGCA ACATACCTT	FAM-CCTCTKCATCCT GCTGCTATGCCTCATC -TAMRA	Previous 95% LOD 24.4 IU/mL using International Standard (genotype A2)[110].
PCR-C	CAACCTCCAAT CACTCACCAAC	ATATGATAAAAC G CCGCAGACAC	FAM-TCCTCCAATTTG TCCTGGTTATCGCT -BHQ1	Previous 95% LOD 2 IU/mL across genotypes A to E[108]. Use of BHQ1 quencher instead of TAMRA.

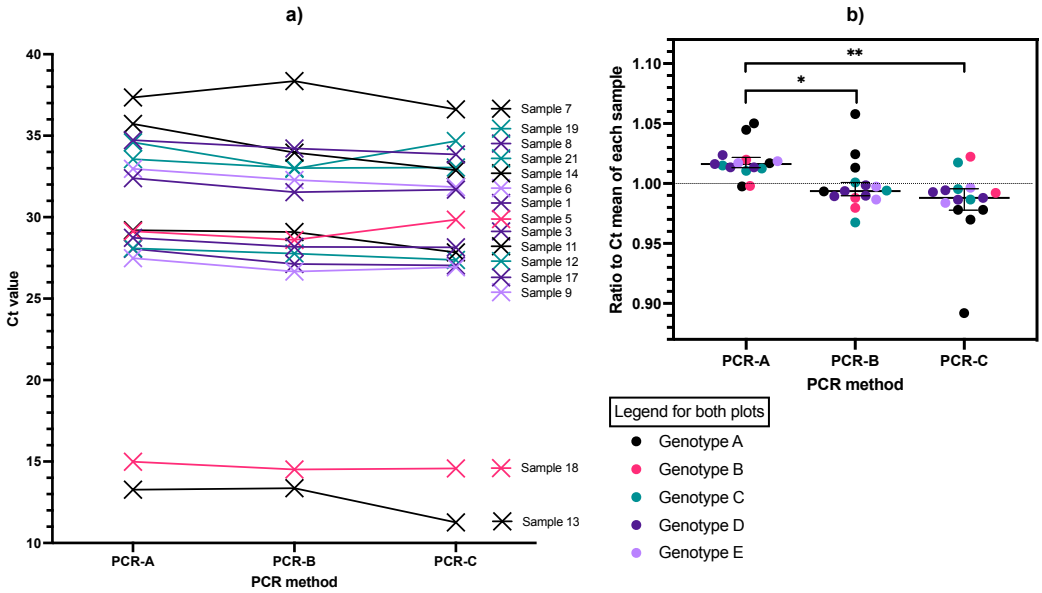
Figure 5.[2] Comparison of Ct values obtained between the PCR primer/probe sets using serial dilutions of three samples of different genotypes: a) Serial 1:5 dilutions of Sample 13 [genotype A2; has no base mismatches with PCR-C], b) Serial 1:2.5 dilutions of Sample 9 [genotype E; sample with base mismatches with PCR-C], c) Serial 1:3 dilutions of Sample 22 [genotype D1; sample with base mismatches with PCR-C]. Negative results are displayed above the dotted lines on each plot. E = PCR efficiency calculated from the slope of the line of best fit for each primer/probe set. R^2 = goodness of fit determined by simple linear regression.



All three assays showed similarly high detection efficiencies for the three samples tested (R^2 >0.999). The sensitivities of PCR-A and PCR-B assays were similar; both showed the same endpoints for Samples 9 and 22, with PCR-B detecting one more dilution of Sample 13. Contrastingly, PCR-C detected two further dilutions of Sample 13 than PCR-B. Despite base mismatches, PCR-C detected one further dilution than the other assays for both Samples 9 and 22. Ct values at each dilution were comparable between assays, except for consistently lower Ct values for Sample 13 in PCR-C. This potentially reflects the effect of the greater degree of probe sequence match to genotype A2 in this PCR.

The comparison was extended to samples of varying genotypes from the control panel; Ct values of each sample tested undiluted were generally comparable between three PCR assays (Figure 6a). When comparing each Ct value to the mean Ct (Figure 2b), the Friedman test revealed significant differences between the methods (Friedman statistic = 12.40, p=0.002). Post-hoc Dunn's showed that when compared to PCR-A, PCR-B (p=0.019) and PCR-C (p=0.003) had increased amplification efficiency across genotypes. There were no significant differences between ratios of Ct values to the means between PCR-B and PCR-C (p>0.999) across all genotypes. However, consistent with previous results, PCR-C had consistently lower Ct values than the mean for genotype A samples (Figure 6b). This partially enhanced amplification efficiency and increased sensitivity, as shown earlier, justified the utilisation of PCR-C for the remaining measurements.

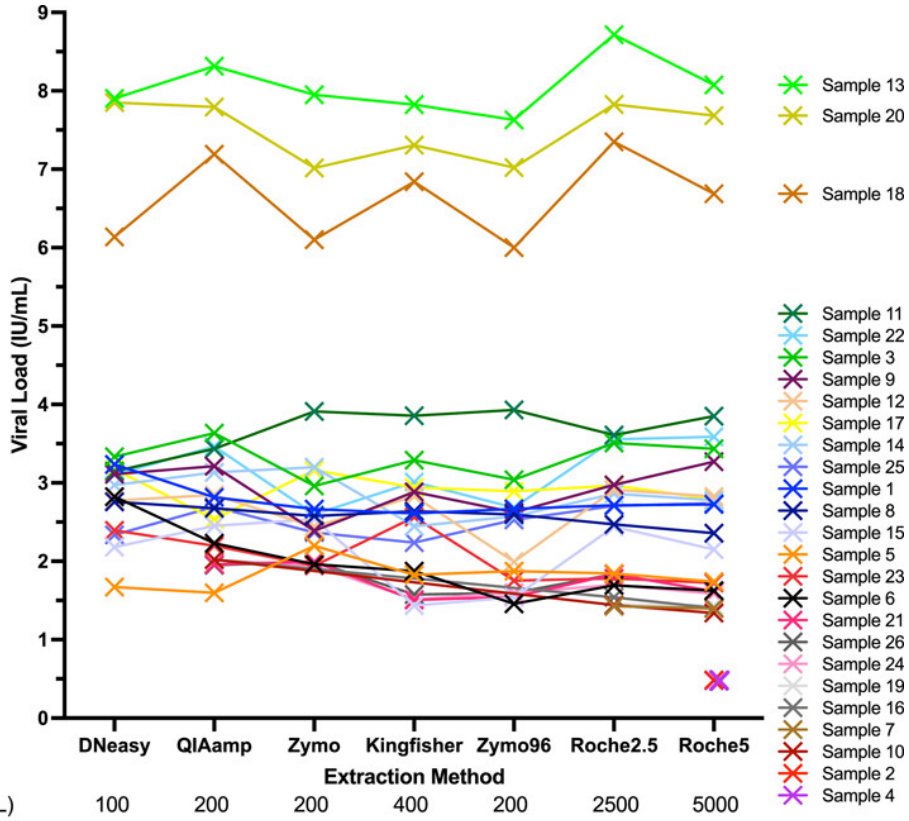
Figure 6.[2] a) Comparison of Ct values in the PCR methods; b) Comparison of PCR methods of the ratio of Ct values for samples to the mean across all methods. Each genotype is categorised in a different colour for both plots, as shown in the legend. The median and interquartile ranges for each method are displayed, with the continuous dotted line showing the Ct mean ratio of each sample as 1.0. * denotes $p \leq 0.05$ and ** denotes $p \leq 0.01$ assessed by post-hoc Dunn's test via Friedman test.



Comparison of DNA extraction methods

Using the preferred PCR-C method, the control panel of 26 samples was further tested to compare the relative sensitivities of seven extraction methods (Figure 7). The VLs detected by methods were similar despite greater copies of HBV DNA detected by larger volume extraction methods. Sensitivities tended to increase with increased sample representation based on sample volume extracted from 17/26 control samples positive with DNeasy (extraction volume: 100 μL; sample representation: 5 μL) increasing to 26/26 samples detected with Roche5 (extraction volume: 5 mL; sample representation: 250 μL; Figure 7) including two samples with very low VL (3 IU/mL; genotypes D and A3). The QIAamp method detected 22/26 samples, despite the small extraction volume of 200 μL and sample representation of 8.33 μL.

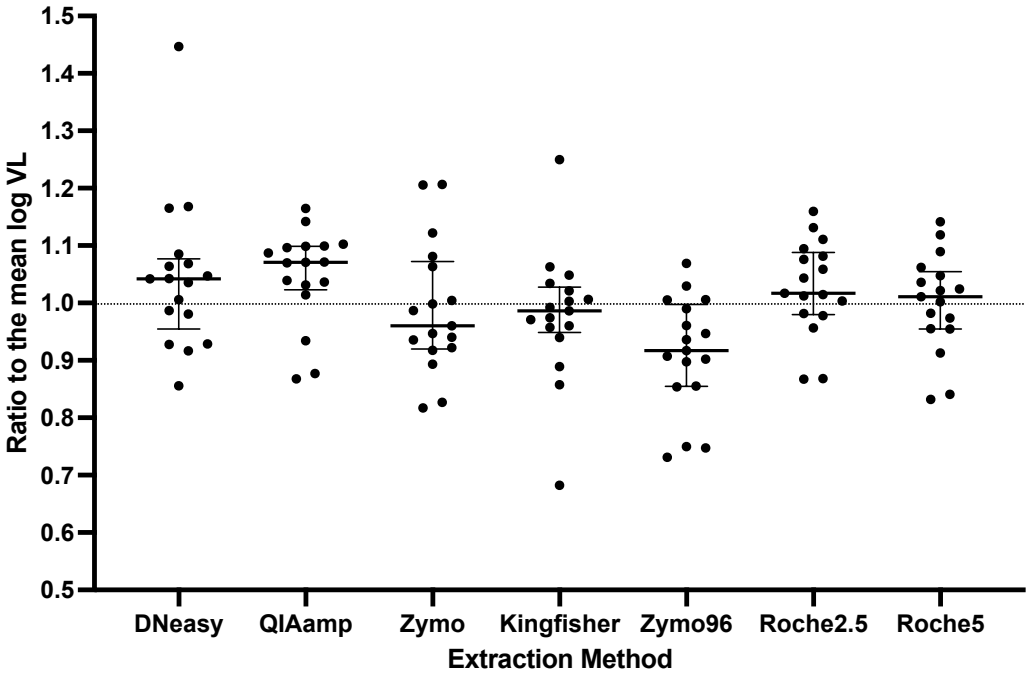
Figure 7.[2] Comparison of calculated viral loads (VL) using seven different extraction methods for each sample. The extraction and elution volumes, volumes used in PCR measurement, and sample representation are displayed below each extraction method. The number of positive control panel samples (n=26) detected by each method is shown. Extraction methods are displayed on the x-axis from smallest to largest sample representation.



Extraction volume (µL)	100	200	200	400	200	2500	5000
Elution volume (µL)	50	60	50	50	20	50	50
PCR volume (µL)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sample Representation (µL)	5	8.333	10	20	25	125	250
Samples detected (out of 26)	17	22	20	20	20	24	26

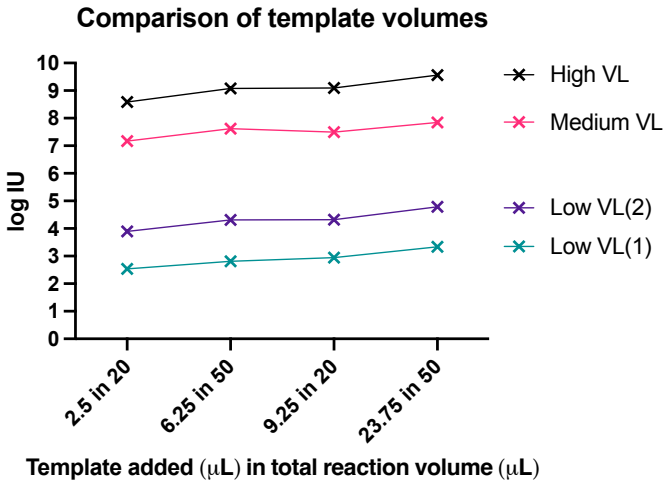
HBV recovery efficiencies were compared for the 17 control samples detected by all methods to further compare the extraction methods. The log-transformed VLs for each sample detected with each extraction method were expressed as ratios to log-transformed VLs across all methods for that sample (Figure 8). The extraction methods provided similar estimates of VL ratios with mean values approximating the overall mean estimate, indicating that increasing sample representation up to 250 μL did not affect the efficiency of HBV DNA recovery. The increased sensitivity and minimal loss in viral recovery supported the use of Roche5 in subsequent measurements.

Figure 8.[2] Differences in log-transformed estimates of VLs derived from each method with the mean VL for the 17 samples with detectable HBV DNA in all extraction methods. The median and interquartile ranges for each method are displayed, with the continuous dotted line showing the log mean of each sample as 1.0. Both graphs display the methods on the x-axis from smallest to largest sample representation.



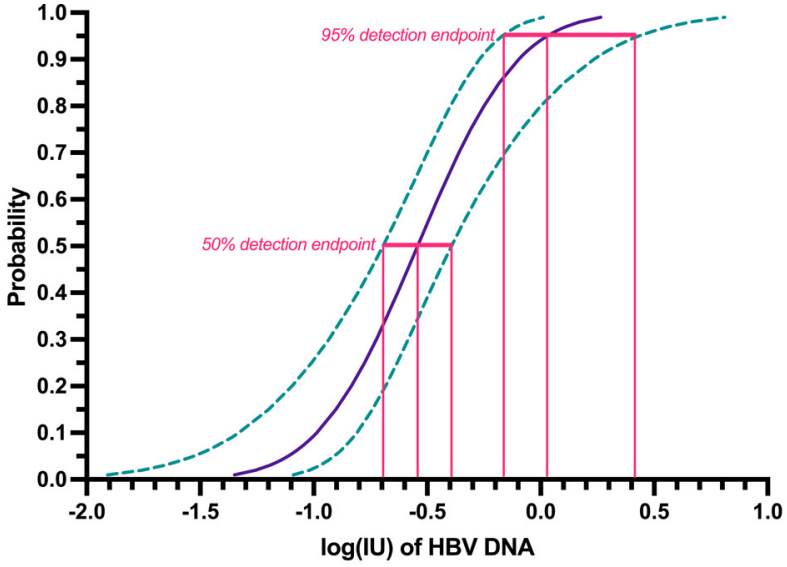
Investigations into increasing sample representation and its effects on HBV DNA detection led to further comparisons of PCR template volumes using control samples of varying VLs. Compared to the original 2.5 µL template volume in a total 20 µL reaction volume used in previous assays, there was a trend toward proportionate increases in amplification by increasing template volumes added to the PCR assay (Figure 9). The necessity to increase sample representation to detect OBI samples characterised by low VL argued for the use of the maximum possible volume of template DNA (23.75 µL using available reagents and consumables) in a total 50 µL reaction volume. This was used in subsequent measurements.

Figure 9.[2] Comparing a range of PCR template volumes and the resulting log IU detected across four samples with varying viral loads (VL).



With this optimal assay developed using Roche5 HBV DNA extraction, PCR-C, and 23.75 μL DNA template, Probit analysis of International Standard dilutions showed the optimal system's 95% and 50% HBV DNA detection endpoints were 0.450 IU/mL (95% CI: 0.285 IU/mL, 1.140 IU/mL) and 0.121 IU/mL (95% CI: 0.085IU/mL, 0.170 IU/mL) respectively (Figure 10). A comparison of our developed system with current NHSBT reference laboratory testing, showed their in-house PCR method to detect HBV DNA in 23/26 samples compared to 26/26 using our optimal system with PCR-C with Roche5. VLs in samples positive in both assays were strongly correlated (r=0.971, p<0.001). The improved sensitivity of the assay presented is further highlighted by the identification of genotype for six additional samples from the control panel compared to UKHSA (Table 1).

Figure 10.[2] Probit analysis to determine the 95% and 50% detection endpoints of the optimal PCR system, showing the probabilities and the corresponding estimated log (IU) of HBV DNA. The ultrasensitive PCR assay developed following extensive testing validations was used for this analysis, using 5 mL extraction method, PCR-C, and 23.75 µL DNA template in the assay. The additional dotted curves represent the 95% confidence limits. Results of the multiple replicates of sequential 1:2 dilutions of the 4th WHO International Standard for HBV DNA from 45.36 IU to 0.09 IU used for Probit analysis are shown below the plot.

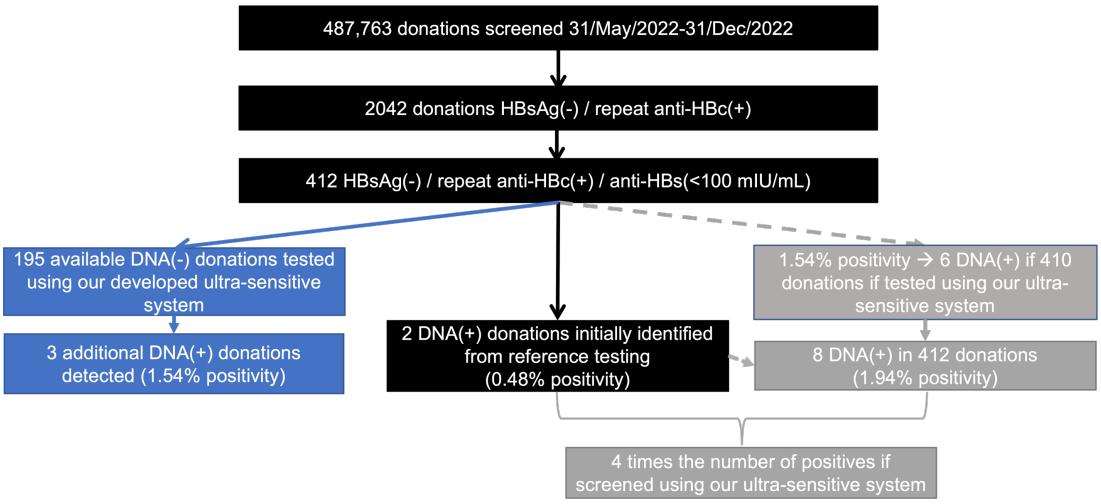


IU	45.36	22.68	11.34	5.67	2.84	1.42	0.71	0.35	0.18	0.09
Observed replicates	3	3	3	12	12	12	12	12	12	12
Detected replicates	3	3	3	12	12	12	11	6	2	2

Application of PCR-C assay for screening anti-HBc positive donors

The developed ultrasensitive PCR system was formally evaluated on recently tested blood donations in England to identify the number of HBV DNA-positive donors deferred through the introduction of anti-HBc screening. Of the initial 487,763 donations screened in 2022, HBV DNA was detected in two out of 412 anti-HBc-positive donations with anti-HBs<100 IU/L by a commercial PCR assay (Figure 11). Testing of the first available 195 anti-HBc-positive donations with anti-HBs<100 IU/L (where the plasma pack was available) identified three further HBV DNA-positive donations, not previously identified by commercial NAT assay in individual testing. One donation showed an average VL of 7.0 IU/mL (first measurement 12.0 IU/mL; repeat measurement 1.6 IU/mL) and was further confirmed via sequencing, assigned as genotype D (Figure 4). The second donation showed an average VL of 1.7 IU/mL (first measurement 1.3 IU/mL; repeat measurement 2.1 IU/mL) but could not be sequenced. The VL for the third donation was not available and could not be sequenced.

Figure 11.[2] Screening and reference lab testing pathway for NHSBT donations since the introduction of anti-HBc screening in May 2022 (black boxes). 195 of 412 confirmed anti-HBc-positive donations with low anti-HBs levels (<100 IU/L) were assayed by ultrasensitive PCR (blue boxes). Extrapolation of HBV DNA detection rate to all 412 HBsAg-negative, anti-HBc-positive, and anti-HBs<100 IU/L donations is shown in grey boxes.



Risk stratification

To analyse potential predictive factors for the presence of HBV DNA in anti-HBc-positive blood donors, the profile of HBV serological markers was compared between the 42 DNA-positive donors and the first 132 DNA-negative donors screened using our ultrasensitive system. HBV VL was generally low; 82% of OBI donors had VLs of less than 10 IU/mL, whereas the highest VL was 101.8 IU/mL. Of donors with genotypes assigned (n=10), 80% were genotype D, one donor was genotype B, and one was genotype A2. There was a significantly higher proportion of anti-HBs-negative donors in the DNA-positive compared to DNA-negative groups (p=0.004) and a trend towards more anti-HBe-negatives in the DNA-positive group compared to the DNA-negative group (p=0.057) (Table 4).

Table 4.[2] Comparison of serological variables between DNA-positive and DNA-negative donors. Fisher's exact test compared all variables.

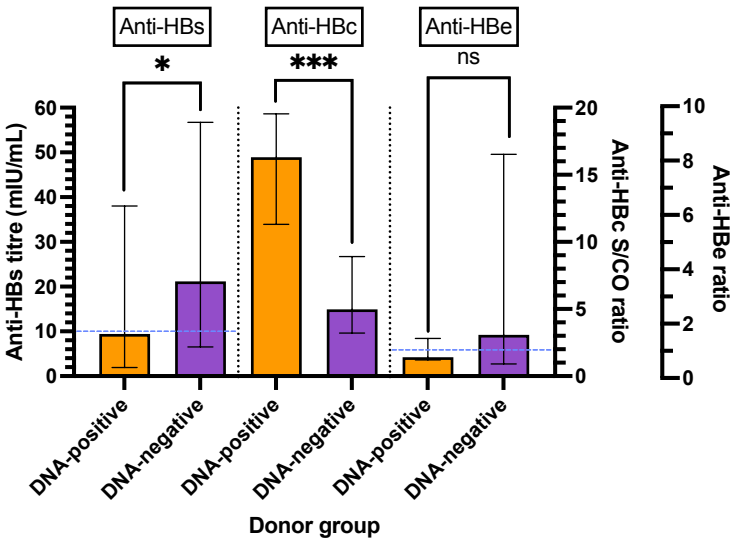
HBV serology marker	*DNA-positive donors (n, %)*	*DNA-negative donors (n, %)*	*p-value*
Anti-HBs-positive	17 (44%)	93 (70%)	0.004
Anti-HBs-negative	22 (56%)	39 (30%)	
Anti-HBe-positive	12 (34%)	68 (53%)	0.057
Anti-HBe-negative	23 (66%)	60 (47%)	

DNA-positive donors had significantly lower median anti-HBs titres (9.4 [1.9-38.0] IU/L) than DNA-negative donors (21.2 [6.5-56.7] IU/L; p=0.040; Figure 12a). Anti-HBc S/CO ratios were significantly higher in DNA-positive donors (16.3 [11.3-19.5]) than in DNA-negative donors (5.0 [3.2-8.9]; p<0.001). Anti-HBe ratios were

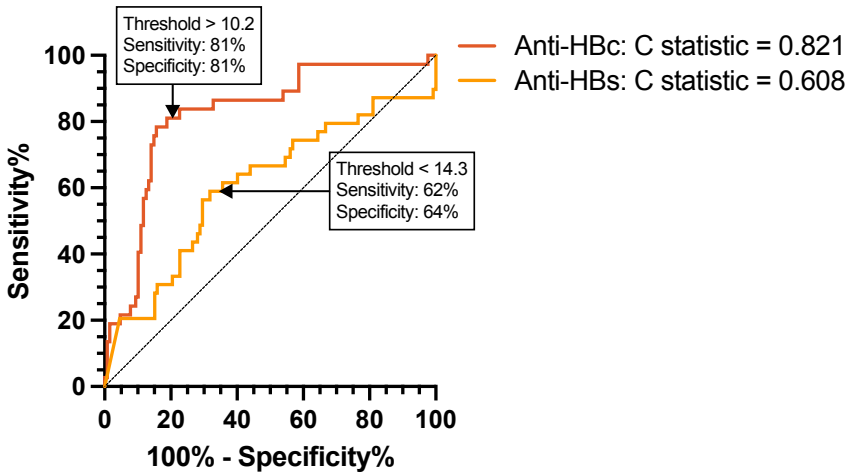
similar between groups (DNA-positive group: 0.7 [0.6-1.4], DNA-negative group: 1.5 [0.5-8.3]; p=0.305). As the anti-HBc and anti-HBs ratios were significantly different between DNA-positive and DNA-negative groups, ROC analyses investigated potential thresholds that would be most sensitive and specific in predicting the presence of DNA (Figure 12b). Consistent with the observed more significant anti-HBc difference compared to anti-HBs, an anti-HBc threshold would be more sensitive and specific than an anti-HBs threshold, with a higher C-statistic. An anti-HBs threshold of 14.3 IU/L would have 62% sensitivity and 64% specificity. Using Architect anti-HBc II (Abbott Diagnostics), a cut-off value of 10.2 for the anti-HBc S/CO ratio would detect 81% of all DNA-positive donations, with 81% specificity. Increasing the sensitivity to 95% would decrease this cut-off to 3.9 but decrease specificity to 41% (Figure 12c).

Figure 12.[2] a) Comparisons of anti-HBs, anti-HBc, and anti-HBe reactivity ratios (based on platform) between DNA-positive donors and DNA-negative donors, where error bars represent medians and interquartile ranges. The dotted blue lines represent the protective cut-off value for anti-HBs (10 IU/L) and anti-HBe (1) where values below are considered negative. * denotes $p \leq 0.05$, *** denotes $p \leq 0.001$ assessed by Mann-Whitney U tests, and ns denotes non-significance; b) Receiver operating characteristic curve (ROC) analysis predicting the anti-HBc and anti-HBs ratio thresholds that would be most sensitive and specific in identifying DNA-positive donations. The dotted line represents the line of identity; c) Anti-HBc S/CO ratios obtained from ROC curve analysis with a range of sensitivities.

a)



b)



c)

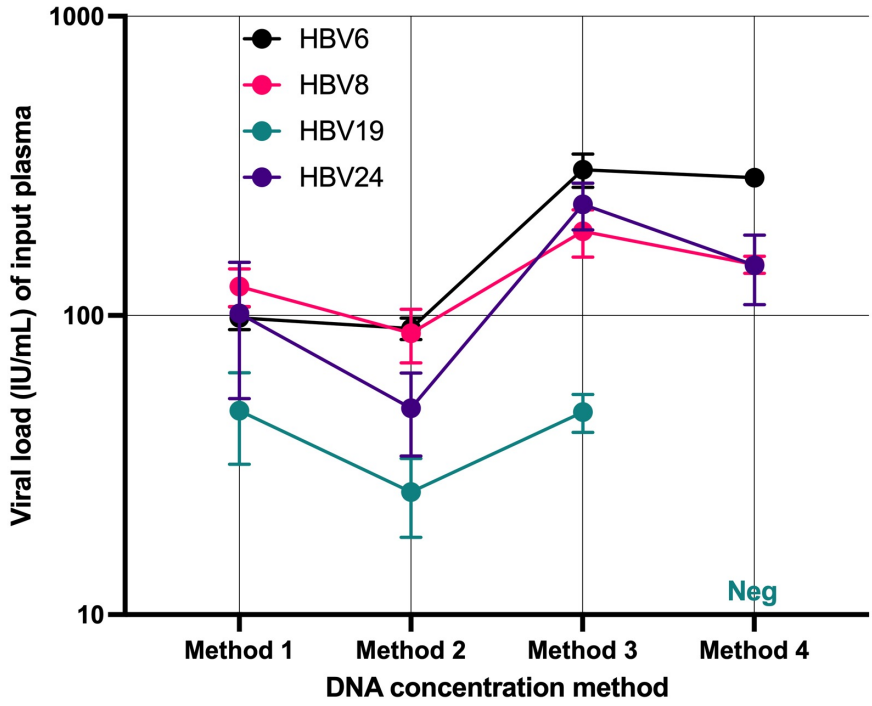
Sensitivity (%)	Specificity (%)	Threshold anti-HBc S/Co ratio
60	88	15.3
68	86	13.2
81	81	10.2
89	46	4.6
95	41	3.9

Efficacy of PEG methods

To compare the efficacy of PEG to concentrate HBV DNA from standard and large-volume extraction methods, 200 µL of plasma containing 96,000 IU of HBV DNA was separately added to 0 mL, 4.8 mL, 9.8 mL and 19.8 mL volumes of HBV-negative plasma, extracted and tested by Methods 1, 2, 3, and 4 respectively, using equal elution and test volumes of extracted DNA from each. Ct values were consistent on replicate PCR testing and comparable between methods, albeit with a modest loss in expected viral recovery when greater input plasma volumes were used (Table 5). These four methods were used to test four non-diluted samples with low VLs (27, 38, 42, and 228 IUs/mL). Taking input plasma volume into account, the estimated VLs were similar between the four methods (Figure 13). One sample provided a negative result using Method 4; this sample was not repeated due to volume limitations, but the entire sample panel was re-concentrated for subsequent measurements.

Table 5.[3] Comparison of Ct values and estimated viral loads obtained in PCR assay replicates between four DNA concentration methods for equal volumes (0.2 mL) of diluted internal control in negative plasma. The same amount of HBV DNA (96,000 IU) was extracted with Method 1 and added to Methods 2, 3, and 4 to extract a total volume of 5 mL, 10 mL, and 20 mL. R = replicate number; VL = viral load; SEM = standard error of the mean.

			PCR assay 1				**PCR assay 2**			
Method	**Testing volume**	**Expected VL (IU/mL)**	R1 (Ct value)	R2 (Ct value)	R3 (Ct value)	**Observed mean VL ±SEM (IU/mL)**	R1 (Ct value)	R2 (Ct value)	R3 (Ct value)	**Observed mean VL (IU/mL) ±SEM**
1	0.2 mL	480,000	27.15	27.21	27.26	442,630 ±10,894	26.92	27.11	27.01	478,012 ±20,977
2	5 mL	19,200	27.23	27.76	27.81	13,287 ±2017	27.12	28.12	27.99	11,279 ±3096
3	10 mL	9600	27.98	28.09	28.23	4444 ±248	29.15	28.97	29.15	1769 ±88
4	20 mL	4800	28.44	28.38	28.40	1743 ±25	28.77	28.78	28.74	1153 ±11



Method	Standard	Standard	PEG	PEG
Volume of input plasma	0.2 mL	5 mL	10 mL	20 mL
PEG added	-	-	2.5 mL	5 mL
Resuspension volume	-	-	1 mL	2.5 mL
Extraction volume	0.2 mL	5 mL	1 mL	2.5 mL
Elution volume	50 µL	50 µL	50 µL	50 µL
Effective test volume	8 µL	200 µL	400 µL	800 µL

Figure 13.[3] Comparison of HBV viral loads obtained from four concentration methods for four samples: standard extraction using 0.2 mL of plasma (Method 1) and 5 mL of plasma (Method 2), polyethylene glycol (PEG) precipitation using 10 mL (Method 3) and 20 mL (Method 4) of plasma. The error bars indicate the mean and standard error of the mean from three PCR replicate measurements. The volume of plasma sample used, PEG, resuspension volumes, extraction volumes, elution volumes and effective test volumes ([volume of input plasma] / [elution volume] * [PCR template volume]) are displayed below the plot. Neg = sample not detected by this method, which could be explained by potential difficulties in large pellet resuspension with the doubling of input volumes than recommended in the manufacturer's protocol.

Viral load comparison using further methods

HBV DNA was detected using real-time PCR in 19/23 samples using Method 1, 22/23 using Method 2, 23/23 using Methods 3 and 4, and 11/12 using Method 5 (p=0.067). When comparing the calculated VLs of the input plasma, HBV DNA detection was proportional to the amount of input plasma (Figure 14A). When comparing across the complete panel of 23 samples, median VLs were within 0.5 log of each other: 283 [89-704 IU/mL] with Method 1, 235 [41-1681] IU/mL with Method 2, 314 [55-3942] IU/mL with Method 3, and 1360 [60-5258] IU/mL with Method 4 (Figure 14B). For the 12 lowest VL samples assayed with all Methods we noted comparable VL across all samples (Figure 15).

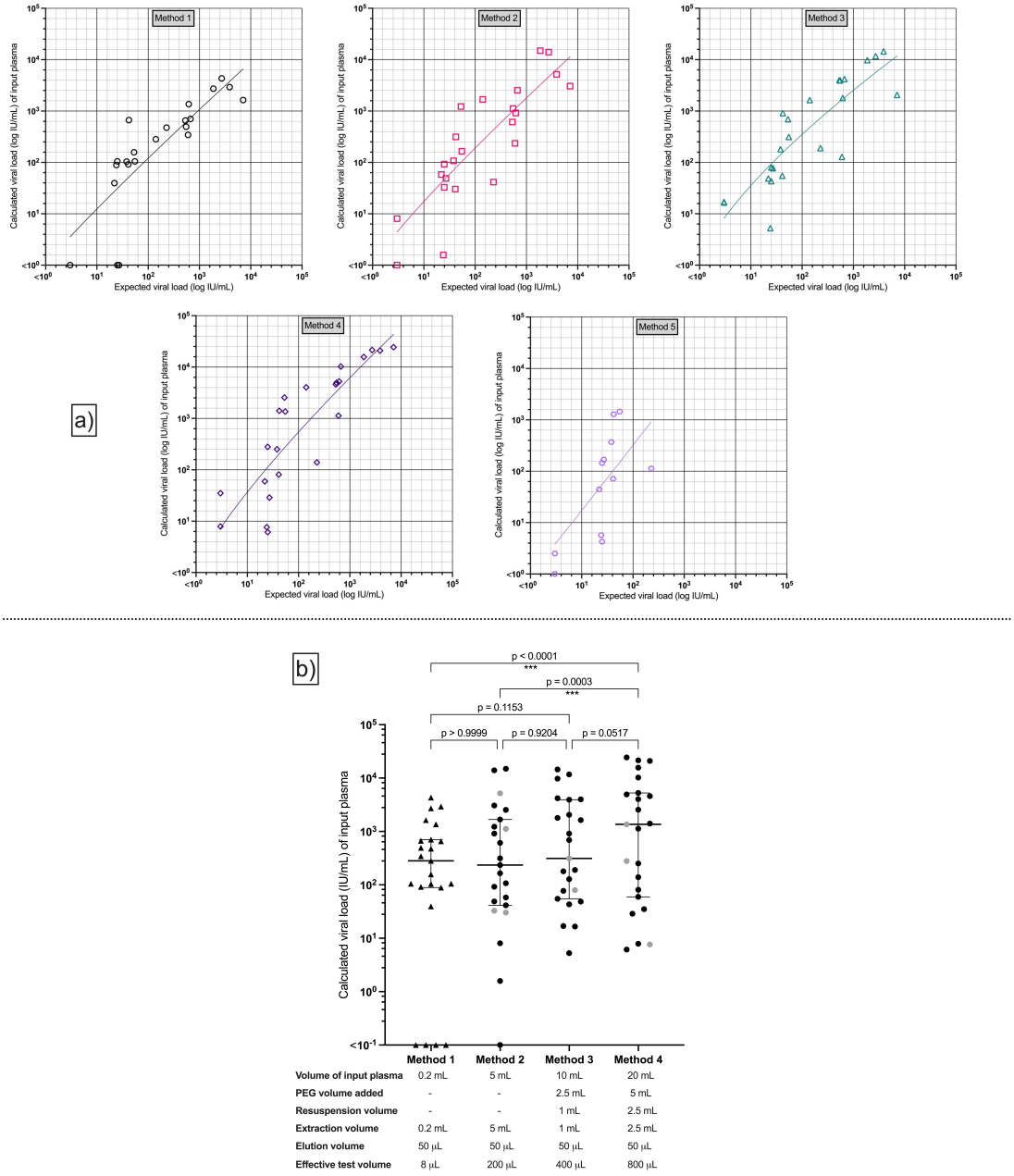
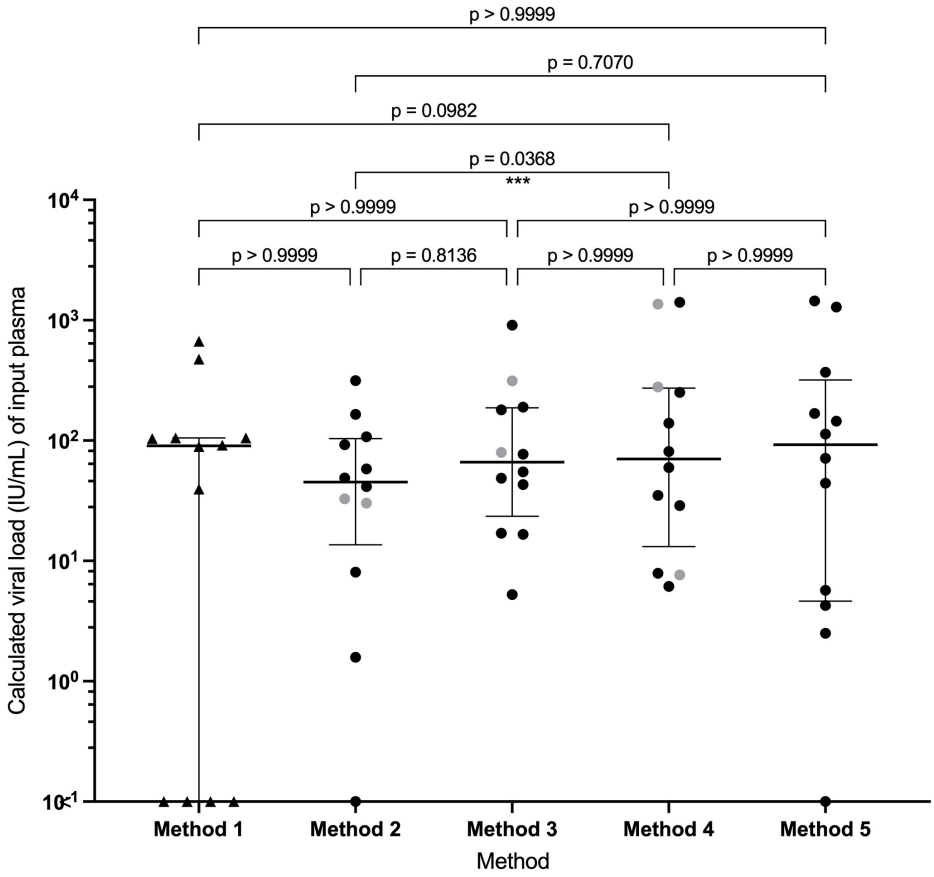


Figure 14.[3] Comparison of viral loads obtained from the five concentration methods: standard extraction without concentration using 0.2 mL (Method 1) and 5 mL of plasma (Method 2), polyethylene glycol (PEG) precipitation using 10 mL (Method 3) and 20 mL (Method 4) of plasma, and ultracentrifugation using 35 mL of plasma (Method 5): A) Comparison of calculated viral loads (normalised to input plasma volume) for each methods, with log-log non-linear regression lines shown and axes drawn to the same scales for each plot. B) Comparison between Methods 1 to 4

(samples were concentrated, extracted, and tested concurrently for Methods 2 to 4) showing calculated viral loads (normalised to input plasma volume) for the set of 23 samples, where the lines indicate the median [interquartile range] for each method. Only the 12 lowest VL samples were assayed through Method 5. The volume of PEG, resuspension volumes, extraction volumes, elution volumes and effective test volumes ([volume of input plasma] / [elution volume] * [PCR template volume]) are displayed below the plot. *P*-values were obtained from the Friedman test with Dunn's multiple comparisons test. Lighter-coloured dots indicate measurements from 9.5 μL extracts instead of 2 μL due to limited extract, as measurements were initially performed with 9.5 μL extracts. IU/mL = international units per millilitre.



	Method 1	Method 2	Method 3	Method 4	Method 5
Volume of input plasma	0.2 mL	5 mL	10 mL	20 mL	35 mL
PEG volume added	-	-	2.5 mL	5 mL	-
Resuspension volume	-	-	1 mL	2.5 mL	1 mL
Extraction volume	0.2 mL	5 mL	1 mL	2.5 mL	1 mL
Elution volume	50 μL	50 μL	50 μL	50 μL	50 μL

Figure 15.[3] Comparison between Methods 0 to 4 (samples were concentrated and extracted concurrently for Methods 1 to 3 and were tested concurrently for Methods 1 to 4) showing viral loads (normalised to input plasma volume) for the 12 samples with the lowest viral loads, where the lines indicate the median [interquartile range] for each method. The volume of PEG, resuspension volumes, extraction volumes, and elution volumes are displayed below the plot. *P*-values were obtained from the Friedman test with Dunn's multiple comparisons test. Lighter-coloured dots indicate measurements from 9.5 μL extracts instead of 2 μL due to limited extract, as measurements were initially performed with 9.5 μL extracts. IU/mL = international units per millilitre.

HBV genome sequencing using further methods

To investigate whether extraction from larger volumes of plasma enhanced the quantity and quality of HBV genome sequencing, DNA extracted by the five methods was amplified by two nested PCRs, and the amplicons directly sequenced. HBV DNA was sequenced in a greater number of samples when input concentration volume increased: 18/23 samples using Method 1, 20/23 using Methods 2 and 3, 23/23 using Method 4, and 11/12 using Method 5 (p=0.234). When detected, all samples were positive in both PCR regions except for samples with lower VL in Method 1, and the sample with the lowest VL (HBV4) which was only positive in the first PCR in two of three methods (Table 6). When detected, amplicon sequences derived from each sample were identical across the four methods, except for one or two nucleotide differences between methods for six samples; these could reflect the natural heterogeneity of the virus populations.

Table 6.[3] A comparison of the sequence completeness (shown by percentage %) and sequence identity of the genome region of length 395 nucleotides derived from amplifying two overlapping genome regions by nested PCR. Sequences assembled from amplicons in the four methods were identical to each other, except for samples with coloured cells. Grey indicates one dissimilar nucleotide from the shaded methods compared to the unshaded methods. Orange indicates two dissimilar nucleotides compared to the other methods. Blue indicates two identical sequences with two dissimilar nucleotides compared to the other methods. '-' indicates where ultracentrifugation was not performed on samples with higher viral loads. 'NS' indicates where sequences were not able to be obtained. * indicates where only one of two amplicons was able to be sequenced. mL = millilitre.

Sample	Viral Load (IU/mL)	Genotype	Method 1	Method 2	Method 3	Method 4	Method 5
11	7115	A	100%	100%	100%	100%	-
22	3881	D	100%	100%	100%	100%	-
3	2715	A	100%	100%	100%	100%	-
9	1871	E	100%	99.7%	100%	100%	-
12	670	C	100%	100%	100%	100%	-
17	619	D	100%	100%	100%	100%	-
14	599	A	100%	100%	100%	100%	-
25	550	D	95.9%	100%	100%	100%	-
1	533	D	100%	100%	100%	100%	-
8	228	D	100%	100%	NS	100%	100%
15	141	E	100%	99.7%	99.7%	100%	-
5	55	B	100%	100%	100%	100%	100%
23	53	A	100%	100%	100%	100%	-
6	42	E	100%	100%	100%	100%	100%
21	41	C	60.5%*	100%	100%	100%	100%
24	38	A	39.5%*	98.5%	99.7%	100%	100%
19	27	C	60.5%*	100%	100%	100%	100%
7	25	A	NS	NS	NS	100%	100%
16	25	A	NS	99.7%	99.5%	100%	100%
26	24	A	NS	100%	100%	100%	100%
10	22	D	60.5%*	100%	100%	100%	100%
2	3	D	NS	NS	NS	92.2%	NS
4	3	A	NS	NS	39.5%*	39.5%*	100%

HBV NGS coverage

Six representative panel samples with a range of HBV VLs from 24-3881 IU/mL were extracted by Methods 2 and 4 (or Method 3 for HBV22 because of sample volume limitations) and assayed through a multi-pathogen targeted capture NGS protocol (Table 7). HBV sequences were obtained from five of the six samples using Method 3/4, but only two of the six using Method 2. Consensus sequences from HBV12, HBV22, and HBV25 were consistent with the sequences derived by nested PCR, with 0, 1, and 3 nucleotide differences in the 395 nucleotide amplicon, respectively. The three samples detected by Method 4 produced 8-95 deduplicated HBV reads. For the two samples that were detected by both methods, nearly two-fold and ten-fold HBV sequences with greater genome coverage were obtained using PEG compared to Method 2 (Table 7). Anellovirus sequences were detected in three samples, Epstein-Barr virus (EBV) DNA was detected in a fourth sample with Method 4, while none were detected in DNA extracted by Method 2. With Method 3 compared to Method 2, greater anellovirus read counts were observed for two samples and greater human herpes virus (HHV)-8 reads for one sample. In summary, the PEG methods greatly enhanced sequence coverage across HBV and other viral genomes (Figure 16).

Table 7.[3] Comparison of deduplicated read counts (dedup rds) and genome coverage proportion (COV) for six selected samples where extracted DNA from Method 2 and Method 4 (or Method 3 for HBV22) were assayed in parallel through a multi-pathogen target capture NGS method. Grey boxes indicate samples run in the method with half-cDNA and half-gDNA, whilst the other three samples were run on a standard cDNA protocol. Anellovirus coverage was not computed as reads may have originated from multiple co-infecting strains or genotypes. HBV = hepatitis B virus; AV = anellovirus; HHV = human herpesvirus; EBV = Epstein-Barr virus.

Sample	Viral load (IU/mL)	Method	HBV		AV	EBV		HHV8	
			Dedup Rds	COV	Dedup Rds	Dedup Rds	COV	Dedup Rds	COV
HBV22	3881	Method 2	254	0.92	-	0	-	-	-
		Method 3	459	0.96	-	3	0.02	-	-
HBV12	670	Method 2	0	-	0	-	-	-	-
		Method 4	95	0.91	11	-	-	-	-
HBV25	550	Method 2	35	0.90	3	-	-	-	-
		Method 4	343	0.99	32	-	-	-	-
HBV1	533	Method 2	0	-	0	-	-	-	-
		Method 4	12	0.41	3	-	-	-	-
HBV7	25	Method 2	0	-	0	-	-	5	0.05
		Method 4	8	0.17	2	-	-	37	0.23
HBV26	24	Method 2	-	-	31	-	-	-	-
		Method 4	-	-	107	-	-	-	-

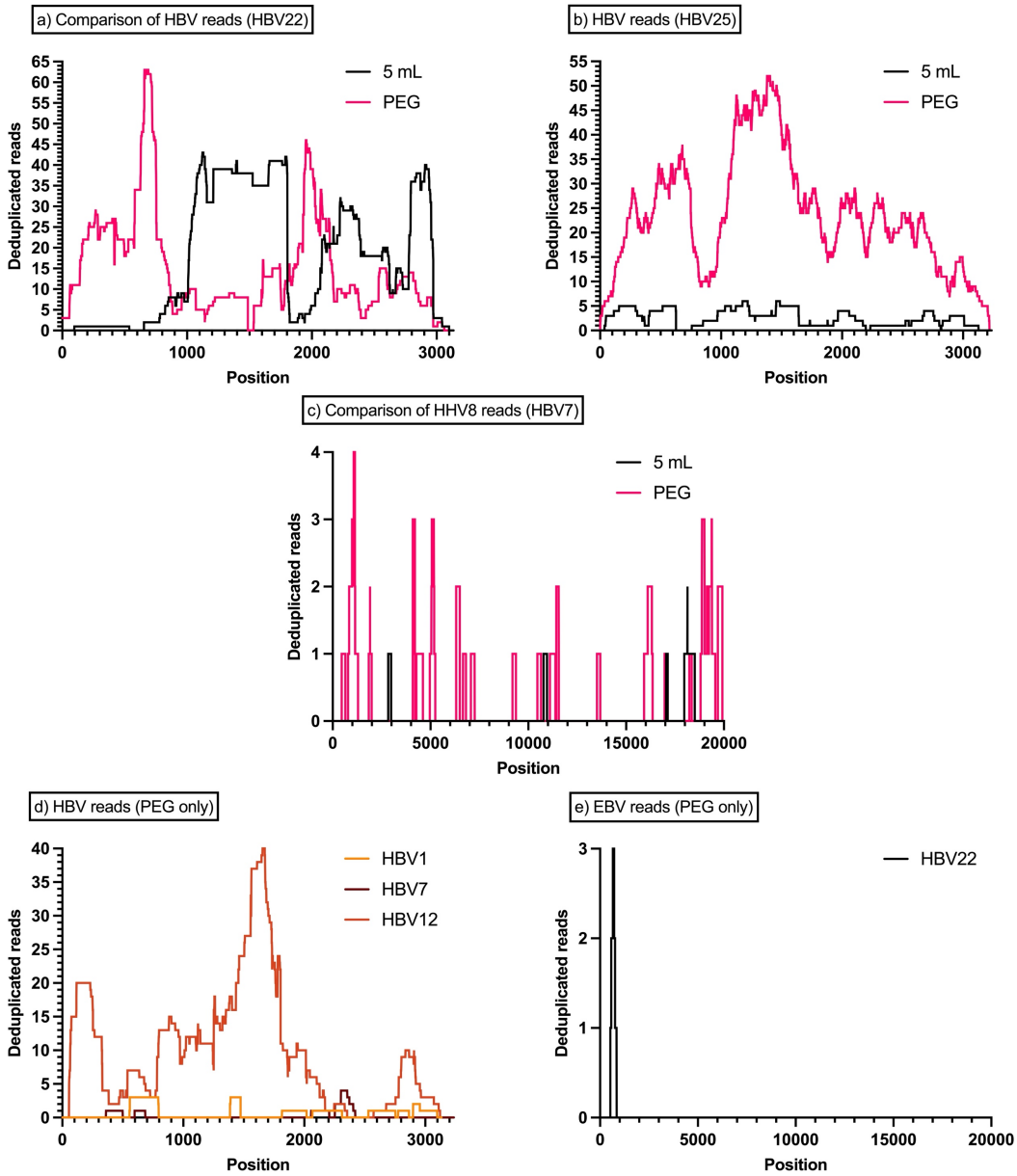


Figure 16.[3] Read depth plots across whole viral genomes for the samples run through the next-generation sequencing protocol. Plots a) to c) show the comparison of genome coverage for HBV and HHV8 detected in samples by both 5 mL (Method 2) and PEG methods (Method 3 or 4), whilst d) and e) show genome coverage for HBV and EBV detected in samples using only the PEG method. Anellovirus coverage is not shown due to viral heterogeneity of the family, hindering the identification of specific genera. HBV = hepatitis B virus; PEG = polyethylene glycol; HHV = human herpesvirus; EBV = Epstein-Barr virus.

Comparison of practicality, costs, and time

Lastly, we compared key factors contributing to the practicality and cost-effectiveness of the different methods (Table 8). The standard extraction method required smaller volumes of plasma and allowed the testing of more samples than PEG or ultracentrifugation. Ultracentrifugation required equipment and technical expertise that may be lacking and costly to implement in standard clinical diagnostic and transfusion laboratories. Hands-on times were similar in the four extraction protocols, but calculated per sample, they would be longer for PEG and longest for ultracentrifugation because of smaller concentration batch sizes.

Table 8.[3] Comparison of different factors between standard extraction, extraction preceded by polyethylene glycol (PEG) precipitation, and extraction preceded by ultracentrifugation methods. NA = not applicable; £ = UK Pounds.

Factor	Method		
	Standard	**PEG**	**Ultracentrifugation**
Principle of concentration	Binding nucleic acids to silica fibre filters	Solution-based chemistry	Sedimentation coefficient
Practicality			
Volume of plasma required	0.2 mL or 5 mL	10 mL or 20 mL	35 mL
Number of clinical samples per run	90 or 21*	10**	6
Technical expertise required	Standard	More than standard (pellet resuspension)	Even more than standard (meticulous sample preparation and pellet resuspension)
Instrument availability	Standard	Standard	Only in certain laboratories
Costs			
Instrument/rotor (approximate upfront cost)	Microcentrifuge for 0.2 mL (£2500); Benchtop megafuge for 5 mL (£7500)	Benchtop megafuge (£7500)	Ultracentrifuge (£90,000 [centrifuge £65,000 plus rotor £25,000])
Tubes needed before extraction	NA	50 mL regular tube (£0.20 each)	Ultra-clear tube (£11.50 each)
PEG Precipitation Kit, cost per sample	NA	£6 for 10 mL £15 for 20 mL	NA
Extraction cost	£3 per sample (for 0.2 mL) £10 per sample (for 5mL)	£7.50 per sample	£7.50 per sample
Approximate times			

Pre-extraction total hands-on time	NA	2 hours	2 hours
Pre-extraction total waiting time	NA (for 0.2 mL) Overnight (for 5 mL)	Overnight	5 hours
Total extraction time	3-5 hours	2 hours	2 hours

*Total 24 for each run, with an additional two negative controls and one low-positive control

**Total 8 for each run, with additional negative control and low-positive control

Discussion

This chapter describes the development of an ultrasensitive PCR system for the detection of HBV DNA; comparisons of extraction methods showed that increased sample representation (sample volume extracted, DNA added to the PCR) contributes proportionally to assay sensitivity. Applying the optimal assay Roche5 and PCR-C, we detected HBV DNA additionally in 1.5% of anti-HBc positive samples that had been negative on standard screening with initial 0.5% HBV DNA positivity rate from those detected with traditional screening methods. This supports the potential utilisation of an ultrasensitive system for HBV DNA quantification in diagnostic practice. Moreover, this study identified anti-HBc antibody levels as predictors of DNA presence in anti-HBc-positive samples.

Using standard calibrated PCR, it was demonstrated that larger extraction volumes translate to improved detection of HBV DNA, consistent with previous studies using in-house[108] and commercial assays[108,116,117]. Previous investigations of low VL samples also found that DNA extraction of 5 mL plasma substantially improved sensitivity compared to smaller and more standard extraction volumes[108]. With a wider range of HBV VLs, the current chapter confirmed this finding where the use of 5 mL extraction volumes enabled the detection of very low VLs and the ability to infer genotypes that were not achieved with other extraction methods. This ability was further improved using PEG precipitation from up to 20 mL of plasma, which was an effective method for concentrating viral DNA and enhancing analyte sensitivity, comparable to ultracentrifugation. The unique properties of the non-toxic and chemically inert PEG solution contribute to its efficacy in detecting and characterising viruses in samples with low VL. By inducing interactions that preferentially crystallise

biological macromolecules such as DNA and virions in the interpolymer spaces between PEG molecules[118], PEG reduces the solubility of viral particles that are concentrated and precipitated from the solvent supernatant. PEG has also been used to enhance HBV e-antigen detection[119], where treating plasma with PEG may also increase the detection sensitivity for novel biomarkers for OBI[1]. Utilising PEG method for screening anti-HBc positive donations may identify a greater proportion of DNA-positives than identified using the Roche5 method but was not performed due lack of time and resources.

Genome sequences of HBV human variants may differ by up to 10%-13% from each other[112] necessitating careful site selection in the design of primers and probes used for HBV DNA NAT to ensure equal sensitivity for the detection of different HBV genotypes. Although the three oligonucleotides in PCR-C were stated to have sequence identity with published HBV sequences[109], there were base mismatches in PCR-C with certain HBV genotypes at two sites in the probe (Figure 3). Despite this, PCR-C showed similarly enhanced amplification efficiency across varying genotypes and assay sensitivity in comparison with PCR-B and PCR-A assays, consistent with the high sensitivity (2 IU/mL) across HBV genotypes of a previously investigated PCR-C-based assay[108]. Results found in this chapter suggest that the mismatches may not have substantially destabilised the primer/probe-DNA duplex, and there was no subsequent decrease in the estimated template quantity. This has been described in other studies investigating mismatches in HIV[120] and influenza[121] that suggested that any effects on PCR product yield were dependent on the positions and nature of the mismatches.

The assay's 95% LOD of 0.450 IU/mL is 10-fold lower than commercial assays utilised in clinical practice to screen blood and organ donors, such as the Grifols (4.5 IU/mL) and Roche (1.4 IU/mL). Considering the effective test volumes, the assay detects more than 2-fold fewer HBV DNA IUs than the Grifols commercial assay (Table 9). Testing of our optimal system on 195 plasma donations identified three further HBV DNA-positive donations that were undetectable with the Grifols assay. This is significant if these were repeat donors and had a risk of HBV transmission in previous donations before the introduction of anti-HBc screening[71]. Given the recently proposed lower limit of VLs associated with infectivity (0.15 IU/mL)[38], the 50% detection endpoint of the current study's assay (0.121 IU/mL) would hypothetically detect and subsequently prevent cases of OBI transmission characterised by extremely low VLs. Although not investigated, this limit of detection is likely to be further improved by utilising PEG precipitation or ultracentrifugation. our study findings add to the limited literature on protocols using PEG precipitation to concentrate viral nucleic acids from human plasma. A previous study showed that concentrating plasma using PEG (using 6 mL and detecting 39.2% of samples) was less efficient than ultracentrifugation (using 12 mL and detecting 54.4% of samples; p=0.079) for the detection of HBV DNA in samples that were non-discriminated reactive from a commercial PCR assay[105]. A further study showed that ultracentrifugation (no volume of serum stated) was marginally superior to PEG precipitation (using 100 microlitres of serum) to concentrate HBV DNA; preparation by ultracentrifugation detected two further DNA-positive samples than PEG[106]. Using much greater volumes of input plasma in both methods and assessing metrics not previously considered, such as VL comparisons and sequencing metrics, this chapter provides a new perspective, suggesting that both methods are similar in their concentration of nucleic acids in low

VL plasma. Our findings highlight the potential applicability of PEG in the transfusion context for clinical virology stakeholders to consider.

Table 9.[2] Comparison of HBV DNA detection in IU between the two commercial methods at NHSBT England and the ultrasensitive PCR, showing the 95% limits of detection and effective test volumes (negating the dead volumes where applicable for the commercial assays – Grifols input 0.800 mL and dead volume 0.300 mL; Roche input 1.300 mL and dead volume 0.450 mL).

Method	*Assay 95% limit of detection (IU/mL)*	*Effective test volume (mL)*	*HBV DNA detected (IU)*
Grifols	4.5	0.500	2.3
Roche ID-NAT	1.4	0.850	1.2
Roche MP-NAT	33.6	0.850	28.6
Ultrasensitive PCR	0.450	2.375	1.069

The DNA positivity rate in the current chapter of 3/195 (1.5%, CI [0.3-4.4]) (Figure 11) is slightly lower than a Swiss study's finding of 7/124 (5.6%, CI [2.3-11.3]; p=0.055) of anti-HBc-positive donors with detectable DNA[100] despite our more sensitive assay. Moreover, that study did not stratify by anti-HBs levels, where blood donations with levels less than 100 IU/L are considered more infectious[38] and more likely to contain HBV DNA. This suggests a lower but still significant positivity rate of OBI donors in England compared to Switzerland, which follows the lower anti-HBc positive prevalence amongst all blood donors[39,100]. Considering the DNA positivity rate in this chapter's cohort, an extrapolated average of 106 [45-213] anti-HBc-positive donations would be DNA-positive per year based on an average of 1,864,484

yearly donations in England from 2009-2018 and an anti-HBc prevalence of 0.30%[39]. Our assay would detect 4-fold more DNA positives than expected from the current screening strategy (Figure 11). However, larger volume extractions with 5 mL are economically costly and labour-intensive for blood services compared to current automated testing. It may be more practical and economical to screen ID-NAT-negative donations with high anti-HBc titres using our ultrasensitive system to increase the likelihood of detecting DNA-positive donations of extremely low VLs. A mitigation strategy could also be utilised, with implementation of anti-HBc donor screening when the prevalence is <2-4%, and, where feasible, the implementation of sensitive HBV NAT when the prevalence is higher[40].

This chapter provides evidence that there is a higher risk sub-group of anti-HBc donors for OBI, irrespective of intermittent viraemia that some might show[69,77]. Anti-HBc titres in sera may reflect the response to HBV core antigens generated and released into the bloodstream by higher levels of intrahepatic replication-competent cccDNA, where a previous study found an association between anti-HBc IgG values with the detection of cccDNA[122]. However, that study found no association in anti-HBc titre with the presence of viraemia[122], contrasting our findings that suggest higher cccDNA levels may also increase HBV virions circulating in the bloodstream. Differences in study populations may explain the discrepancy, where the current chapter had living blood donors with a smaller proportion of anti-HBs-positives compared to deceased, mostly anti-HBs-positive, liver donors in the previous study[122]. Additionally, the previous study's small population of six DNA-positive donors[122] decreases validity compared to the 42 in the current chapter. Moreover, the higher proportion of anti-HBs-negativity in the DNA-positive versus DNA-negative donors

found in our study might be due to immune complexes formed between anti-HBs and HBV particles[82], which are removed from circulation and reduce measurable HBV DNA VL. A previous study also found the highest HBV DNA detection frequencies in anti-HBc-positive, anti-HBs-negative subjects[70].

Our findings support the use of anti-HBc and anti-HBs titres as serological biomarkers complementary to NAT to better define non-infectious donors without DNA. An anti-HBc titre cut-off of 10.2 (Architect anti-HBc II) that could identify OBI donors with detectable DNA who have increased risks of hepatocellular carcinoma[123]. This supports previous suggestions in immunocompromised populations that a combination of high anti-HBc titre and low anti-HBs titre would identify those at increased risk of HBV reactivation[124]. The use of these biomarkers may help minimise the number of donors deferred by positive anti-HBc screening results but whose donations represent a low risk of HBV transmission. This may also support the recruitment of donors with rarer blood types who typically immigrate from HBV-endemic areas[83], and is especially important for HBV-endemic countries where the prevalence of anti-HBc is high and universal anti-HBc screening would be unsustainable[82]. However, while anti-HBc and anti-HBs titres were predictive of viraemia detection on ROC analysis (Figure 12b), the association was imperfect (<100% specificity) and there were indeed HBV DNA-positive samples among samples with low anti-HBc levels.

Our investigations of serological correlates of DNA positivity were performed on single time-point donations. OBI is associated with fluctuating DNA levels and donors may not invariably be NAT positive even when using the most sensitive assays

at a single time-point. Furthermore, increasing assay sensitivity renders testing outcomes susceptible to limiting dilution effects. For samples with very low VLs, the stochastic partitioning of HBV sequences according to the Poisson distribution requires repeat testing of multiple replicates to confirm an initial low-level positive result, which is laborious and unsuitable for HBV screening of donors in real-time. Nucleotide sequencing of amplicons generated by nested PCR may provide reassurance of positivity if the amplified HBV DNA sequence is distinct, but this assay is similarly prone to limiting dilution effects and may possess reduced sensitivity compared to the screening assay.

Despite larger volumetric concentration factors, both PEG and ultracentrifugation methods present limitations. Practical barriers exist for obtaining and processing large volumes of samples in routine clinical practice outside the blood donation setting. Indeed, limited plasma volume restricted the repetition of experiments in this study, such as for samples that produced false-negatives. The detection failure of one sample using ultracentrifugation could be explained by the difficulty of resuspending small and often translucent pellets. Potential lack of reproducibility would affect the detection of very low VL, where full pellet resuspension is important. Further, large pellets produced by the 20 mL modified PEG protocol present difficulties in resuspension, which may have mediated the non-detection of one sample in the feasibility experiment.

In conclusion, the improved sensitivity of an ultrasensitive PCR combined with a large-volume extraction can detect a greater proportion of blood donations positive for HBV DNA from anti-HBc-positive donors. While the recently introduced anti-HBc

screening in England provides the means to effectively identify and exclude potentially infectious donors with OBI, its specificity is low and leads to substantial wastage of donations that possess negligible HBV transmission risk. Blood services may consider introducing more sensitive HBV DNA testing for anti-HBc-positive donors, along with surrogate markers of host response (such as anti-HBc titres) that may enable more effective risk stratification for infectivity, perhaps enabling the current extensive deferral of donors to be reversed. Future studies could investigate the performance of PEG precipitation on more standard and available volumes of plasma. Additionally, whilst the detectability and genotyping of HBV DNA using sensitive PCR methods is necessary, using next-generation sequencing to obtain more genomic data would improve whole-genome assembly and provide a more accurate representation of the viral genome.

Chapter 2: Next-generation sequencing of hepatitis B virus at low viral loads

Most of this chapter is based on the paper under review (as of September 2025):

Fu MX, Perdomo MF, Lumley SF, et al. Next-Generation Sequencing Methods for Sensitive Characterisation of Hepatitis B Viral Genomes: A European Multicentre Study, medRxiv, June 2025.

Introduction

The advent of high-throughput sequencing platforms in the mid-2000s, coined 'next-generation sequencing' (NGS), has made dramatic advances in the characterisation of host and pathogen genomes in health and disease[125]. Metagenomics, a commonly used NGS method to sequence all genomic material in a sample, has been instrumental in detecting a wide range of microbes, surveillance of emerging infections, and identifying novel pathogens[126,127]. Compared to Sanger sequencing where sub-genomic amplicons spanning regions of interest are sequenced, the ability to sequence complete microbial genomes using NGS in an unbiased manner has proven to be a powerful tool[126,127]. The wider adoption of NGS in clinical microbiology and public health has been hindered by complex methodological workflows, complications in the detection and reporting of contaminating sequences, and a reduced sensitivity compared to real-time and nested PCR assays[126,128]. In particular, the inadequate sensitivity of untargeted metagenomics has limited the detection of HBV, where many HBV-infected clinical samples contain low copy numbers of the viral genome[129,130].

HBV can be classified into 10 genotypes (A to J) based upon sequence divergence ≥8%, with inter-genotypic differences in geographic distribution, transmission mode, and clinical outcomes[131]. Sequencing full-length HBV genomes can help infer accurate (sub)genotypes and recombinants, better understand mechanisms of transmission and persistence, identify treatment-resistant and immune-escape mutations, and develop new therapeutics[132]. Furthermore, whole-genome sequencing (WGS) of low VL samples might enable the characterisation of OBI and the assessment of viral clearance and reactivation, and may be progressively relevant to the field of functional cure.

Methodologies for sequencing viral genomes need to be time- and cost-efficient, specific, and sensitive[133]. A comprehensive evaluation of different methodologies is vital to inform their wider uptake in clinical, public health and research laboratories. Previous studies have been limited to individual workflows and laboratories, with few coordinated comparisons. When protocols were compared, studies often used synthetic materials with lower sample diversity due to limited clinical sample availability[134]. A comparable analysis of the effectiveness of NGS methods for the characterisation of hepatitis C virus was conducted almost a decade ago[135]. Another comparison only investigated samples with relatively high VLs and did not provide information on the detection limits of different NGS methods[134].

Amid calls for the HBV field to work progressively toward developing NGS methods[131], this chapter brought together six European laboratories with nine established virus sequencing protocols, encompassing four commonly used NGS methods. First, the untargeted metagenomics method sequenced all genomic material

in the samples. The second method involved the addition of biotinylated nucleic acid probes to enrich for large numbers of known pathogens and selectively capture target sequences before Illumina sequencing. The final methods involved PCR amplification of HBV fragments, followed by sequencing using the short-read Illumina or the long-read Oxford Nanopore Sequencing (ONT) platforms. The latter three methods have previously been shown to be sensitive for sequencing HBV genomes[127,130,133,136].

The four NGS methods were evaluated using a panel of 23 HBV DNA-positive plasma samples with low VLs. We compared their utility in detecting and generating HBV sequences and co-detecting other virus species.

Materials and methods

Samples

The twenty-three lowest VL large-volume plasma samples from the panel described in Chapter 1 were included. To reflect the heterogeneity in the extraction methods used by NGS protocols and account for the non-negligible stochastic effect of measuring low VLs, geometric mean VLs were calculated across eight separate real-time PCR measurements from eight extractions (five of which were obtained from Chapter 1 and three further extractions from 5 ml of plasma). The samples had geometric mean VLs ranging from 0.2 to 6207 IU/ml. Furthermore, a control plasma sample, negative for HBV markers and other screened blood-borne infections, was obtained from NHSBT. All 24 samples, including the negative control, were sent to each laboratory on dry ice, with anonymised sample identifiers and no other sample information.

Sequencing methods

The panel of 24 blinded samples was used to evaluate the performance of nine established NGS protocols at six expert European laboratories. Methods included two untargeted metagenomic protocols using Illumina (MTG-A and MTG-B), three pan-viral probe-based enrichment protocols using Illumina (TAC-A, TAC-B, and TAC-C), two HBV-specific PCR amplification protocols using Illumina (PCR-A and PCR-B [PCR-Illumina]) and two HBV-specific PCR amplification protocols using ONT (PCR-1 and PCR-2 [PCR-ONT]). Nucleic acid extractions were performed at individual laboratories, except for three protocols for which extractions were conducted separately in the coordinating laboratory in Oxford (PCR-A, PCR-B, PCR-

2). The input plasma volumes for extractions ranged from 0.2 to 20 ml (Table 11). Extracted nucleic acids underwent library preparation according to local protocols, where input volumes ranged from 5 to 50 µl. Effective test volumes ranged from 4 to 800 µl. Sequencing platforms and average sequencing depth per sample differed between methods (Table 10). Full details of protocols are found in Appendix 1. The pre-capture libraries for TAC-B were sequenced for MTG-B; thus, the samples were prepared similarly for both protocols.

Table 10.[4] Protocol details of the NGS protocols analysed, including the sequencing workflows and analysis pipelines (until the point of genome assembly) for each method. Effective test volume was calculated as [library prep input volume]/[extraction output volume]*[extraction input volume], where TAC-A, TAC-B and MTG-B also accounted for reductions in effective test volume through cDNA synthesis, and PCR volumes were also accounted for in PCR-based protocols. Numerical superscripts next to method identifiers indicate where the same laboratory performed the protocols.

	MTG-A	**MTG-B[1]**	**TAC-A[1]**	**TAC-B[1]**	**TAC-C**	**PCR-A**	**PCR-B[2]**	**PCR-1**	**PCR-2[2]**
Technology	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	Illumina	ONT	ONT
Pre-extraction concentration	No	PEG Virus Precipitation	No	PEG Virus Precipitation	No	No	No	No	No
Nucleic acid extraction	QIA-symphony DSP Virus/Pathogen Mini Kit	Roche Large Volume Kit	Roche Large Volume Kit	Roche Large Volume Kit	QIAamp DNA Mini Kit	Roche Large Volume Kit*	Roche Large Volume Kit*	Roche Large Volume Kit	Roche Large Volume Kit*
Input/output volume	200 µL / 60 µL	20,000 µL / 50 µL	5,000 µL / 50 µL	20,000 µL / 50 µL	400 µL / 60 µL	5,000 µL / 50 µL	5,000 µL / 50 µL	5,000 µL / 50 µL	5,000 µL / 50 µL
Pre-prep PCR amplification (volume of extract added, total reaction volume)	No	No	No	No	No	Yes Round 1: 5 µL, 25 µL Round 2 (x4): 1 µL, 25 µL	Yes 2x (7 µL, 25 µL), followed by clean-up into 20 µL	Yes 2x (5 µL, 25 µL)	Yes, 2x (7 µL, 25 µL), followed by clean-up into 20 µL
Library prep input volume	26 µL	5 µL	5 µL	5 µL	50 µL	5 µL (0.2 ng/µL)	8.3 µL	10 µL	8.3 µL
Effective test volume	87 µL	800 µL	350 µL	800 µL	333 µL	~4 µL	581 µL	200 µL	581 µL
rRNA depletion	No	No	No	No	No	No	No	No	No
Human DNA depletion	No	No	No	No	No	No	No	No	No
cDNA synthesis	No	Yes	Yes	Yes	No	No	No	No	No

Library prep kit	NEBNext Ultra II FS (DNA)	Twist (cDNA)	Twist (half DNA & half cDNA)	Twist (cDNA)	Roche Hyperprep (DNA)	Nextera XT (DNA)	NEBNext Ultra II FS (DNA)	SQK-RBK114.96 (DNA)	SQK-NBD114.96 (DNA)
Probe capture	No	No	Yes	Yes	Yes	No	No	No	No
Random amplification cycles	N/A	16	16	16	30 (2x 15)	12	0	30	0
Average gigabases (Gb) or megabases (Mb) sequenced per sample	5.03 Gb	208.2 Mb	49.6 Mb	105.1 Mb	2.5 Gb	317.3 Mb	47.4 Mb	2.2 Mb	312.7 Mb
Platform	NextSeq 2000	NovaSeq X	NovaSeq X	NovaSeq X	NovaSeq X	MiSeq V2	NovaSeq X Plus	MinION	MinION
Sequencing strategy	Paired end 150	Paired end 150	Paired end 150	Paired end 150	Paired end 150	Paired end 250	Paired end 150	N50: 1.06 kb	N50: 600 bp
Read filtering	nf-core taxprofiler (MetaMix was used for detecting additional viruses)	Kraken2, FastQC, Trimmomatic, BWA-mem2, custom script for aggregating reads by reference organism, samtools, viralconsensus	Castanet	Castanet	TracesPipe	FASTQC, Trimmomatic, Smalt	QUASR, CutAdapt, Skewer, Bowtie2	See Appendix 1.	dorado, hbv-fieldbioinformatics
Assembly method	Reference-based mapping	Reference-based mapping	Reference-based mapping	Reference-based mapping	Bowtie 2 and metaSPAdes	IVA, de novo using custom scripts	Reference guided to closest reference	Minimap2	Reference guided to closest reference

*These extractions were performed separately in the central coordinating centre, and eluates (or spin columns before the elution step) were sent to the laboratories instead of plasma

Factors relating to the technical practicality and costs associated with the use of NGS assays greatly influence their adoption for clinical diagnostics. Laboratories were asked to provide the total time and cost of each protocol step from extraction to sequencing on a standardised template document, considering the typical number of samples that would normally be included per run for each protocol. Times for each stage were separated into per-person labour time and waiting times (which included machine running times), and costs were standardised using a 1:0.90:0.75 USD:EUR:GBP exchange rate, as of May 18, 2025.

Bioinformatic processing

Post-read filtering and genome assembly, either mapping to the closest available reference or *de novo*, were performed using established protocol-specific pipelines at each centre (Table 10). After deduplication of reads or removal of PCR duplicates and processing of sequences to begin at the EcoR1 restriction site (the conventional origin of the genome[137]), CSV files for each assembled genome/sample were sent from each centre, containing the number of each base called at each nucleotide site. These files were received at the coordinating laboratory, where further bioinformatics analyses were performed using a common set of tools previously described[135]. A 95% majority base consensus sequence was calculated at each genome position possessing one or more base reads, incorporating the calling of ambiguous bases when necessary. Constructed assembled sequences for each sample were examined and realigned to ensure consistent numbering, compare nucleotide similarities, and assess genome coverage and accuracy. Within-sample consensus sequences were assembled for each sample from the different NGS protocols by

combining more than one similar assembled sequence with genome coverage of around 100% using the program Sequence Join in the SSE package[113], followed by visual inspection of sites with ambiguous bases. Specific sequences from each method were compared with the within-sample consensus, and the numbers of nucleotide differences were recorded using the program Sequence Dist[113]. Missing bases were not included in comparisons of sequence identity. Whilst a threshold of one base read per nucleotide site allowed for representation of all called bases in all protocols regardless of background contamination or sequencing errors, a more stringent minimum threshold of 10 base reads at each nucleotide site was also applied; the re-assembled sequences were re-aligned to ascertain potential differences when utilising a higher threshold. Whilst this stringent threshold was applied, such a high threshold is not advocated for most targeted enrichment or untargeted metagenomics purposes of low VL samples.

Nested PCR and Sanger sequencing

Sequences of a portion of the surface gene obtained from Chapter 1 were included for comparison to the NGS sequences. Using DNA extracted using Roche5, nested PCR and sequencing of two further regions were performed: one spanning the surface and polymerase genes, and another spanning the X and precore/core genes (Table 11). Within-sample consensus NGS sequences were genotyped as described in Chapter 1. When whole-genome assemblies could not be constructed (i.e. no two complete NGS sequences were similar), genotypes were defined from either the most complete NGS sequence from an individual protocol or the Sanger sequences obtained from Chapter 1, the latter if there were no similar NGS sequences.

Table 11.[4] Details of primers used for sequencing of HBV amplicons, indicating the start and end nucleotide positions of the amplified regions. Outer primer sets are provided in the above cell, while inner primers are indicated in the below cell. Nucleotide positions are numbered according to the D00330.1 reference sequence.

Region	**Primer sequence (5' to 3')**	**Nucleotide positions of amplified region**	**Length of region**
S	CATCAGGAYTCCTAGGACCCCT; GAGGCATAGCAGCAGGATGMAGAGG; GACTTCTCTCAATTTTCTAGGGG; AGTAAACTGAGCCAAGAGAAACGG	219-375 & 400-637	395 (157 & 238)
	CGTGTTACAGGCGGKGTKTTTCTTGT; ATGATAAAACGCCGCAGACACATC; GATGTGTCTGCGGCGTTTTATCAT; ACGGACTGAGGCCCACTCCCATAG		
SP	ATCCMGAYTGGGACYTCAA; CGTTGCCKDGCAACSGGGTAAAGG	3212-969	973
	TCATCCTCAGGCCATGCAGT; GACACACTTTCCAATCAATNGG		
XC	TCTTGCCCA AGGTCTTACAT; TCCCACCTTATGAGTCCAAG	1673-2370	698
	ATAAGMGGACTMTTGGACT; CAGCGAGGCGAGGGAGTTCTTCTT		

Definitions

The following terms in this chapter have been defined to aid readability.

- Protocol: unique individual NGS workflows performed by individual laboratories.
- Method: a collection of similar protocols based on sequencing technology and platform. Methods included untargeted metagenomics followed by Illumina sequencing (MTG), targeted metagenomics using probe capture followed by Illumina sequencing (TAC), PCR amplification of HBV before Illumina sequencing (PCR-Illumina), and PCR amplification of HBV before ONT sequencing (PCR-ONT).
- Genotype: distinct genetic variations of HBV based on nucleotide differences of ≥8%.
- Subgenotype: subgroups within specific HBV genotypes differing by 4-8% in viral genome sequence and distinct from the broader HBV genotypes.
- Strain: a sequence that differed by >5% from other sequences from the same sample, indicating a different (sub)genotype.
- Reference sequence: a known digital HBV DNA sequence used as a standard for genome assembly.
- Assembled sequence: individual sequences constructed from individual protocols, which underwent read filtering and assembly via protocol-specific bioinformatic pipelines, followed by calculation of majority base consensus sequence at the coordinating laboratory and realignment of assembled sequences where necessary.

- Within-sample consensus sequence: a more robust complete genome sequence for each sample, derived from the most commonly observed nucleotide at each genome position across available assembled sequences from all protocols for the same sample.
- Contamination/contaminating sequence: a genetically different sequence and strain from the within-sample consensus sequence or other assembled sequences, where applicable (>5% nucleotide differences).
- True-positive: similar to other sequences from the same sample (<5% nucleotide differences).
- False-positive sequence: detection of an HBV DNA sequence in the negative control.
- HBV-specific bases read/reads: the total number of base reads at all nucleotide positions, after deduplication of sequencing reads and PCR duplicates, where applicable.
- Genome coverage: the proportion of the viral genome with consensus bases called.
- Shannon entropy: a measure of the diversity of base counts in a sequence, which may result from technical sequencing errors or natural variability within infecting viral populations and could also be influenced by VL. Shannon entropy value ranges from 0 (no variability) to 1 (equal frequencies of all bases).

Detection of other virus species by PCR

Since HBV was not the only possible virus sequenced from the five TAC and MTG protocols from three laboratories, the deduplicated read counts and genome coverage percentages were obtained for any additional viruses detected from the sample panel, following the full bioinformatics pipelines for each centre. When a certain virus was detected by more than one NGS method in the same sample, confirmatory PCR was performed in all samples.

Multiplex real-time PCR was performed to detect all human herpesviruses (HHV) as previously described[138]. Real-time PCR for human polyomavirus 6 (HPyV6), JC polyomavirus (JCPyV), and Merkel cell polyomavirus (MCPyV) was performed as previously described[139]. Detection of anelloviruses followed a nested PCR protocol to amplify alphatorquevirus, betatorquevirus, and gammatorquevirus separately.

For anellovirus, a previously described protocol[140] was modified by using GoTaq G2 DNA Polymerase (Promega) in a total 25 µl reaction volume with 5 µl of extracted DNA (extraction protocol using 5 ml of plasma[2]) in the first-round reaction and 2 µl of first-round template in the second-round reaction. Further, the annealing temperature was modified to 58ºC and the number of cycles during second-round amplification was altered to 35. Samples positive for the specific torqueviruses by nested PCR underwent confirmatory secondary PCR using SYBR Select Master Mix (Thermofisher Scientific). Reaction volumes were 20 µL, with 2 µL of first-round template with 200 nM primers. Cycle conditions were 95ºC 5 min, 40 cycles of 95°C

15 sec, 58°C 1 min, and a dissociation curve. A random selection of amplicons was also sent for Sanger sequencing as previously described[2].

Statistical analyses

Spearman's correlation test deduced the significance of the association between VLs and multiple parameters, where α was 0.05. Data analyses and visualisation were performed with GraphPad Prism (v10.4.2, LLC).

Results

HBV detection and accuracy of assembled sequences

HBV amplicons were obtained for up to 23/23 samples by Sanger sequencing, with good concordance between sequences generated by Sanger and NGS sequencing. Within-sample consensus NGS sequences were constructed for 14/15 samples with VL above 50 IU/ml (Table 12). Sequences assembled using different NGS protocols were generally identical or nearly identical to the within-sample consensus. A few individual sequences were >5% divergent from the within-sample consensus (shown in pink), indicating a different contaminating HBV strain. Assembled sequences were compared for eight samples with lower VLs below 130 IU/ml, for which within-sample consensus sequences were unavailable. Different HBV strains were detected by at least one protocol (>5% divergent from the others) for all eight samples. Three samples, VLs 1.1, 1.8, and 8.5 IU/ml, yielded discrepant sequences in all NGS protocols.

Table 12.[4] Overview of the detection of HBV in sample sets, when majority consensus bases were called at a threshold of 1 base per nucleotide site. The percentage genome completeness is given in each cell. The left-hand side of the table with blue headers shows the sequences obtained from the NGS methods, whilst the right-hand side (calamine colour) shows the sequences obtained by Sanger sequencing (length of S amplicon = 395 nucleotides; SP = 973 nucleotides; XC = 698 nucleotides). Within-sample consensus sequences were assembled for each sample from the individual sequences of the NGS methods that had around 100% genome coverage and were genetically similar, which were assigned to HBV genotypes. The percentage genetic relatedness of the sequences from each method was compared to the within-sample consensus sequence, where assembled sequences similar to the within-sample consensus were shaded on a grayscale, and different strains (differing in >5% nucleotide sequence from the within-sample consensus) were shaded in a pink scale. The absence of sequences was shaded in black. Where within-sample consensus sequences could not be assembled (i.e. no two complete sequences were similar), genotypes were defined from Sanger sequences, and the NGS sequences were instead compared to one another. Samples are ranked from highest to lowest geometric mean viral load. For TAC-B: library preparation failed for sample HBV13; samples HBV4, HBV9, and HBV19 had reads of poor quality for the original pipeline to process, so the mean base quality was dropped below 1.0. #: For PCR-1, a negative water control was tested instead of the negative plasma control. Alphabetical superscripts (a, b, c) represent identical sequences. Numerical superscripts next to method identifiers indicate where the same laboratory performed the protocols.

		PCR (Nanopore)		PCR (Illumina)		Target capture			Metagenomics				Sanger sequencing		
Sample	**Viral load (IU/mL)**	**PCR-1**	**PCR-2[1]**	**PCR-A**	**PCR-B[1]**	**TAC-A[2]**	**TAC-B[2]**	**TAC-C**	**MTG-A**	**MTG-B[2]**	**Within-sample consensus**	**Genotype assigned**	**S**	**SP**	**XC**
HBV15	**6207.0**	100%	100%	91%	100%	11%	100%	90%	25%	72%	**Y**	**A2**	12%	30%	22%
HBV8	**3154.0**	100%	100%	98%	100%	91%[c]	97%	99%	50%	14%	**Y**	**D1**	12%		
HBV2	**3108.0**	100%	91%		100%	90%[c]	81%	100%		25%	**Y**	**A2**	12%		22%
HBV7	**1058.0**	100%	100%	98%	99%	17%	83%	93%	11%	25%	**Y**	**C1**	12%	29%	22%
HBV19	**859.8**	100%	49%		100%	22%	4%	88%	10%		**Y**	**D3**	12%	31%	22%
HBV16	**599.2**	100%	85%	59%	100%	9%		75%			**Y**	**A1**	12%		
HBV1	**551.6**	100%	86%	76%	100%	6%	38%	88%	4%		**Y**	**D2**	12%		
HBV14	**522.3**	100%	100%	92%	100%	9%	95%	80%		49%	**Y**	**E**	12%	30%	
HBV23	**337.1**	100%	86%	99%	100%	64%	71%	93%		16%	**Y**	**D4**	12%	31%	22%
HBV17	**299.9**	100%	100%	96%	92%		14%	50%			**Y**	**E**	12%	30%	
HBV9	**138.1**	100%	100%	33%	99%	9%	8%	35%	2%		**Y**	**A1**	12%	30%	
HBV5	**128.4**	100%	35%		89%			66%			**N**	**E**	12%	30%	
HBV22	**73.6**	100%	83%		86%			54%			**Y**	**D2**	12%	31%	
HBV4	**60.5**	100%	85%	59%	100%	35%	7%	100%	12%		**Y**	**B4**	12%	30%	22%
HBV13	**60.4**	100%	85%		98%			91%	5%		**Y**	**D1**	12%	31%	22%
HBV21	**41.0**	100%	68%[a]		74%		7%	43%			**N**	**A**	12%	30%	
HBV6	**11.3**	100%	33%		90%		3%	28%		3%	**N**	**D3**	12%	31%	
HBV18	**8.5**	60%	67%		88%						**N**	**A**	12%		
HBV20	**3.3**	100%	82%[a]		81%			26%	<1%		**N**	**D**	12%		
HBV12	**1.8**	15%	67%		97%			25%			**N**	**A**	12%		
HBV24	**1.1**	99%	33%[b]		88%						**N**	**E**	12%		
HBV11	**0.3**	100%	68%		85%						**N**	**D**	12%		
HBV3	**0.2**	71%	68%		91%			5%			**N**	**A3**	5%		
Negative (HBV10)	**-**	100%[#]	67%[b]	48%	88%						**N**	**-**			

Within-sample consensus	Shading key					
Y (% sequence identity to within-sample consensus)						
	No sequence	≥85%	≥90%	≥95%	≥98%	≥99.5%
N (similarity of sequences)						
	No sequence	Different sequence to others	Similar sequence			

All four PCR-based protocols detected false-positive HBV sequences in the negative control (genome coverage, 48%-100%), which were identified as different strains. The four protocols also detected numerous contaminating sequences in other samples, including those with low VL (<10 IU/ml). These four protocols were performed in three different laboratories, except for extraction, which the coordinating centre conducted for three of the four protocols. Some contaminants and false positives matched sequences of other *ex vivo* viruses (Table 12), but most were non-identical. For PCR-based methods other than PCR-1, the proportion of contaminants or the lack of detection at the lowest VLs deterred the ability to ascertain true-positive detection confidently. TAC methods had very few contaminating sequences, and no contaminating sequences were assembled from MTG methods. Utilising a 10-base instead of a 1-base threshold for calling consensus bases did not affect the detection of contamination in the negative control. However, the higher threshold eliminated the detection of contaminants in other samples for PCR-1, reduced the detection of contaminants for PCR-2 (including reduced genome coverage), reduced the genome coverage for lower VL samples for PCR-B, and eliminated the detection of contaminants in TAC-C (Table 13).

Table 13.[4] Overview of the detection of HBV in sample sets, when majority consensus bases were called at a threshold of 10 bases per nucleotide site. Otherwise, same legend as Table 12.

		PCR (Nanopore)		PCR (Illumina)		Target capture			Metagenomics				Sanger sequencing		
Sample	**Viral load (IU/mL)**	**PCR-1**	**PCR-2[1]**	**PCR-A**	**PCR-B[1]**	**TAC-A[2]**	**TAC-B[2]**	**TAC-C**	**MTG-A**	**MTG-B[2]**	**Within-sample consensus**	**Genotype assigned**	**S**	**SP**	**XC**
HBV15	**6207.0**	100%	100%	91%	100%	5%	100%	80%		12%	**Y**	**A2**	12%	30%	22%
HBV8	**3154.0**	100%	99%	98%	100%	91%[b]	97%	99%			**Y**	**D1**	12%		
HBV2	**3108.0**	100%	90%		100%	90%[b]	81%	100%			**Y**	**A2**	12%		22%
HBV7	**1058.0**	100%	100%	98%	99%	11%	83%	59%			**Y**	**C1**	12%	29%	22%
HBV19	**859.8**	100%	49%		100%	22%	4%	44%			**Y**	**D3**	12%	31%	22%
HBV16	**599.2**	100%	85%	59%	100%	9%		9%			**Y**	**A1**	12%		
HBV1	**551.6**	100%	50%	76%	100%	6%	22%	66%			**Y**	**D2**	12%		
HBV14	**522.3**	100%	85%	92%	100%	9%	95%	59%		9%	**Y**	**E**	12%	30%	
HBV23	**337.1**	100%	57%	99%	98%	50%	71%	82%			**Y**	**D4**	12%	31%	22%
HBV17	**299.9**	100%	100%	77%	60%		14%				**Y**	**E**	12%	30%	
HBV9	**138.1**	100%	100%	33%	99%	9%	8%				**Y**	**A1**	12%	30%	
HBV5	**128.4**	100%			57%			8%			**N**	**E**	12%	30%	
HBV22	**73.6**	100%	54%		77%						**Y**	**D2**	12%	31%	
HBV4	**60.5**	100%	85%	59%	100%	35%	7%	89%			**Y**	**B4**	12%	30%	22%
HBV13	**60.4**	100%	85%		94%			49%			**Y**	**D1**	12%	31%	22%
HBV21	**41.0**	100%	37%		73%[c]		7%				**N**	**A**	12%	30%	
HBV6	**11.3**	100%			57%		3%				**N**	**D3**	12%	31%	
HBV18	**8.5**		37%*		33%*						**N**	**A**	12%*		
HBV20	**3.3**	100%	17%		43%[c]						**N**	**D**	12%		
HBV12	**1.8**		17%		56%[c]						**N**	**A**	12%		
HBV24	**1.1**				71%[c]						**N**	**E**	12%		
HBV11	**0.3**	99%	4%		29%[c]						**N**	**D**	12%		
HBV3	**0.2**		52%		74%[c]						**N**	**A3**	5%		
Negative	**-**	100%[#]	17%	48%	45%						**N**	**-**			

Within-sample consensus	Shading key					
Y (% sequence identity to within-sample consensus)						
	No sequence	≥85%	≥90%	≥95%	≥98%	≥99.5%
N (similarity of sequences)						
	No sequence	Different sequence to others	Similar sequence			

Overall, true-positive HBV sequences of >5% genome coverage were detected in 5-6/23 samples using MTG, 10-17/23 samples using TAC, 9-13/23 samples using PCR-Illumina, and 16-19/23 samples using PCR-ONT protocols. MTG-B detected 7/13 samples at lower coverage and read numbers, which were detected by TAC-B, including the six samples with the highest coverage detected by TAC-B; samples for both protocols were prepared in the same run before the capture step.

HBV read depths and genome coverage

The highest number of HBV-specific bases was read in PCR methods (up to 10^9), followed by TAC (up to 10^7) and the lowest in MTG (up to 10^4). A strong correlation was observed between nucleotide counts and VL using the PCR-ONT and TAC methods, whereas this correlation was moderate when using the PCR-Illumina and MTG methods (Figure 17A). Base counts were similar between samples for the PCR-Illumina methods. For PCR-ONT, bases obtained for true-positive samples with VL <50 IU/ml ranged from $1x10^4$ to $5x10^7$. For TAC and MTG methods, base counts were much lower for samples with lower VL. The approximate threshold to reliably detect the sample panel with true-positive sequences was >50 IU/ml or $>10^6$ bases for PCR-ONT methods, >50 IU/ml or $>10^4$ bases for TAC methods, >100 IU/ml or $>10^7$ bases for PCR-Illumina methods, and >3000 IU/ml or $>10^3$ bases for MTG methods. Utilising a 10-base instead of a 1-base threshold for calling consensus bases failed to detect HBV in the majority of samples for MTG methods, and removed the need for a VL threshold to detect most of the sample panel with true-positive sequences for TAC and PCR-1 methods (Figure 17B).

Figure 17A.[4] Relationship between viral loads and HBV-specific bases read for each group of methods on a common log scale, when majority consensus bases were called at a threshold of 1 base per nucleotide site. Pink indicates contaminating sequences, including where HBV sequences were obtained for the negative HBV10 sample on the y-axis. Samples not detected are found on the x-axis. Spearman's correlation test deduced the significance of the association between viral loads and base counts (R^2 value and *p*-values listed in boxes). HBV: hepatitis B virus; MTG: untargeted metagenomics; TAC: target capture; ONT: Oxford Nanopore Technologies.

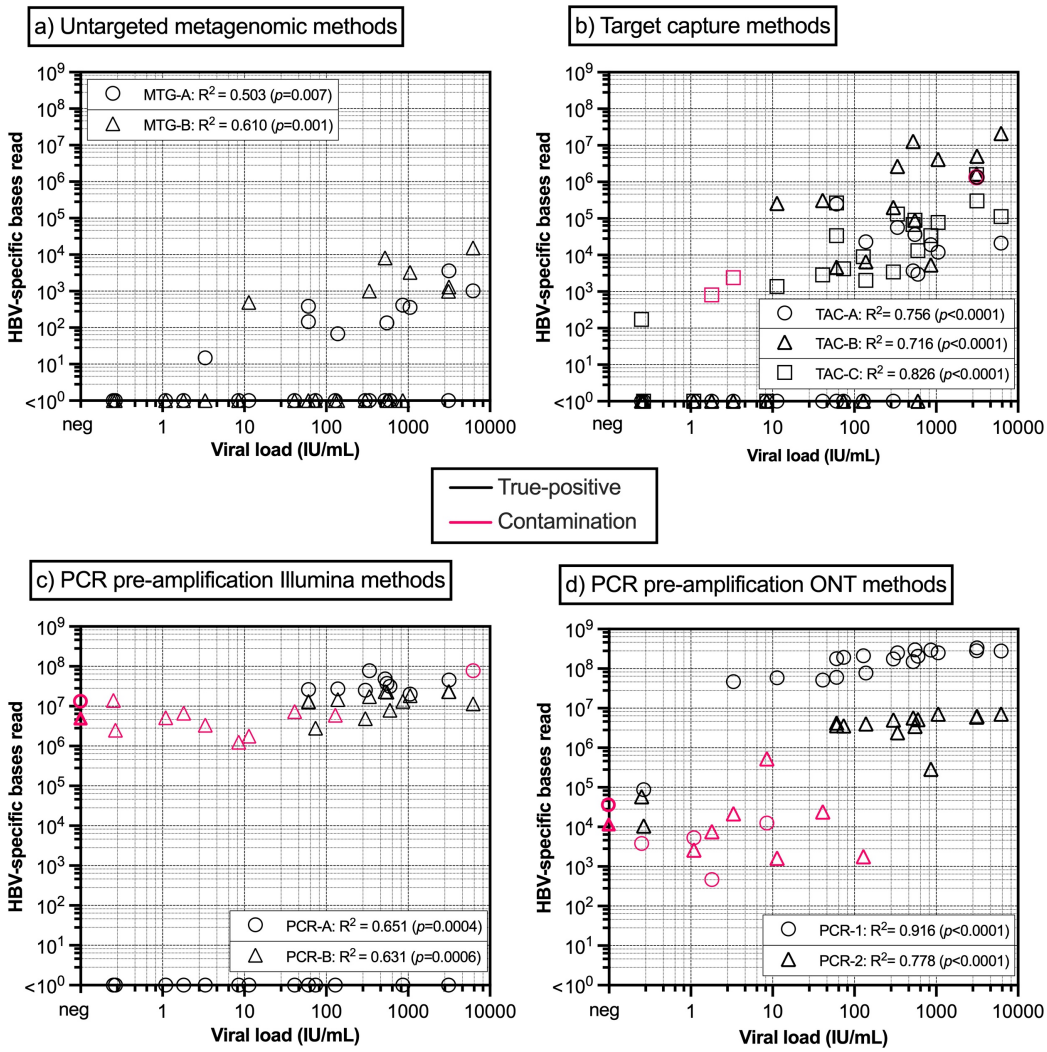
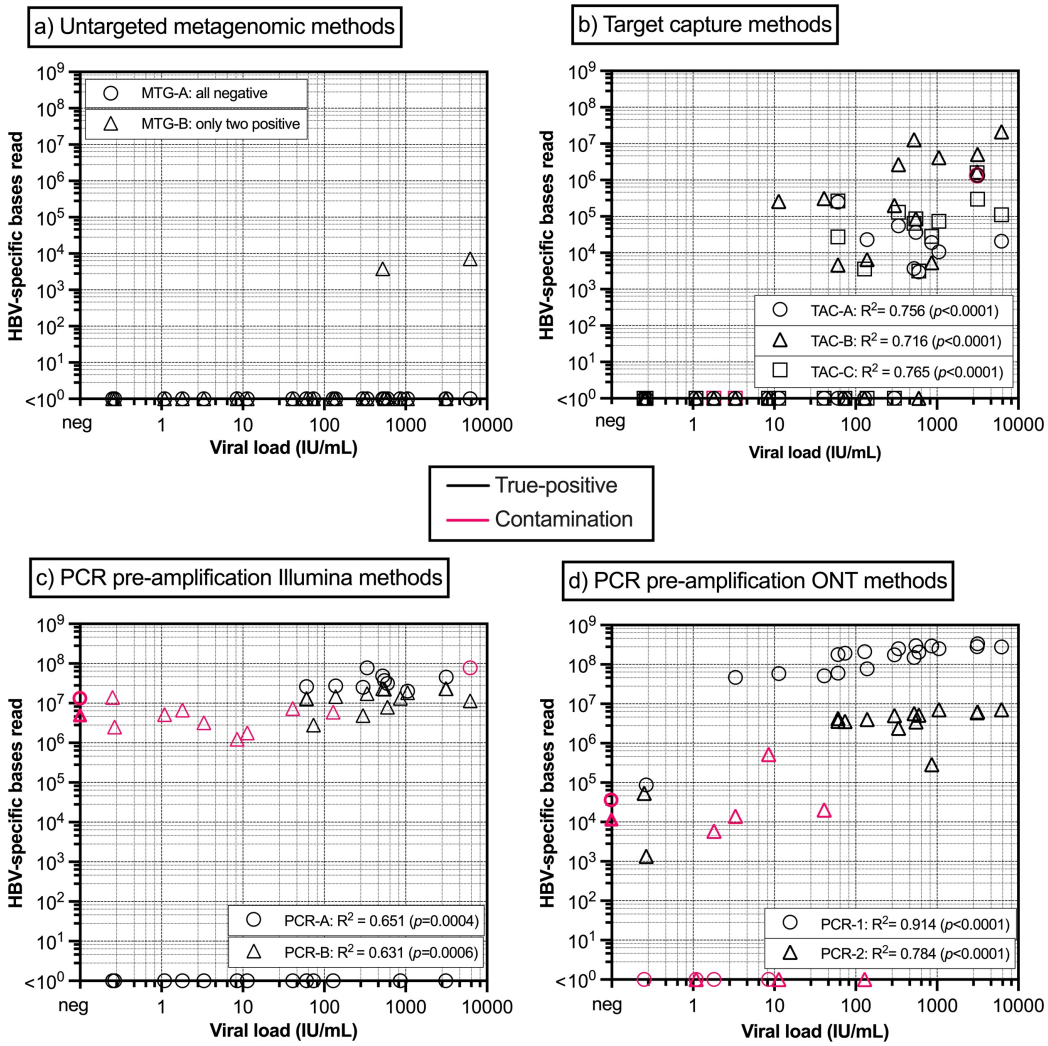


Figure 17B. Same as Figure 17A but applying a 10-base threshold.



Following multiple alignment of read assemblies, the completeness of assembled sequences was analysed. With a minimum coverage threshold of 1 for calling consensus bases and excluding contaminants, complete or near-complete genome sequences were assembled for all samples with VL >10 IU/ml for PCR-1 protocol, >200 IU/ml for PCR-B, >1000 IU/ml for TAC-C, and in none of the samples for MTG methods (Figure 18A). Sub-analyses using a minimum threshold of 10 bases found that coverage thresholds of 80% and 40% could be applied to exclude contamination in the PCR-Illumina and PCR-ONT methods, respectively (Figure 18B).

Figure 18A.[4] Relationship between viral loads and breadth coverage of HBV assembled sequences for each group of methods on a common axes scale, when majority consensus bases were called at a threshold of 1 base per nucleotide site. Pink indicates contaminating sequences, including where HBV sequences were obtained for the negative HBV10 sample on the y-axis. Samples not detected are found on the x-axis. Spearman's correlation test deduced the significance of the association between viral loads and genome coverage (R^2 value and *p*-values listed in boxes). HBV: hepatitis B virus; MTG: untargeted metagenomics; TAC: target capture; ONT: Oxford Nanopore Technologies.

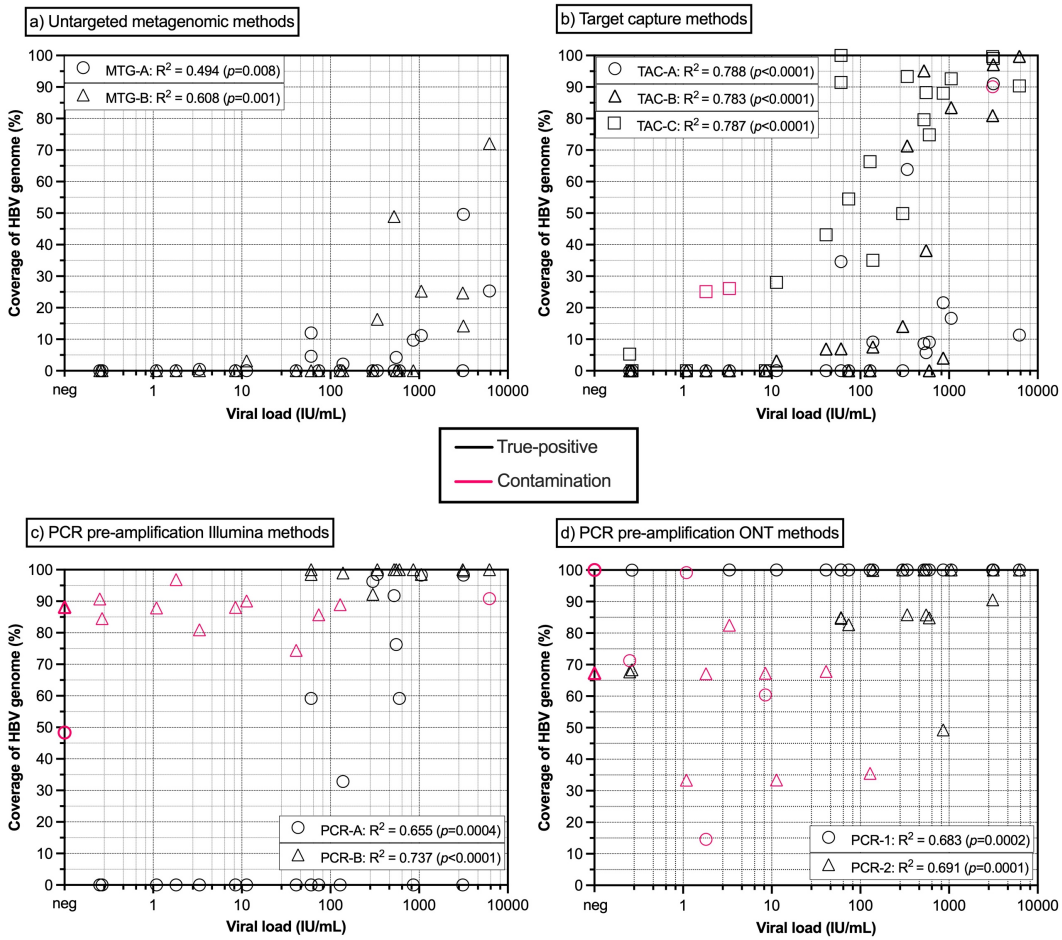
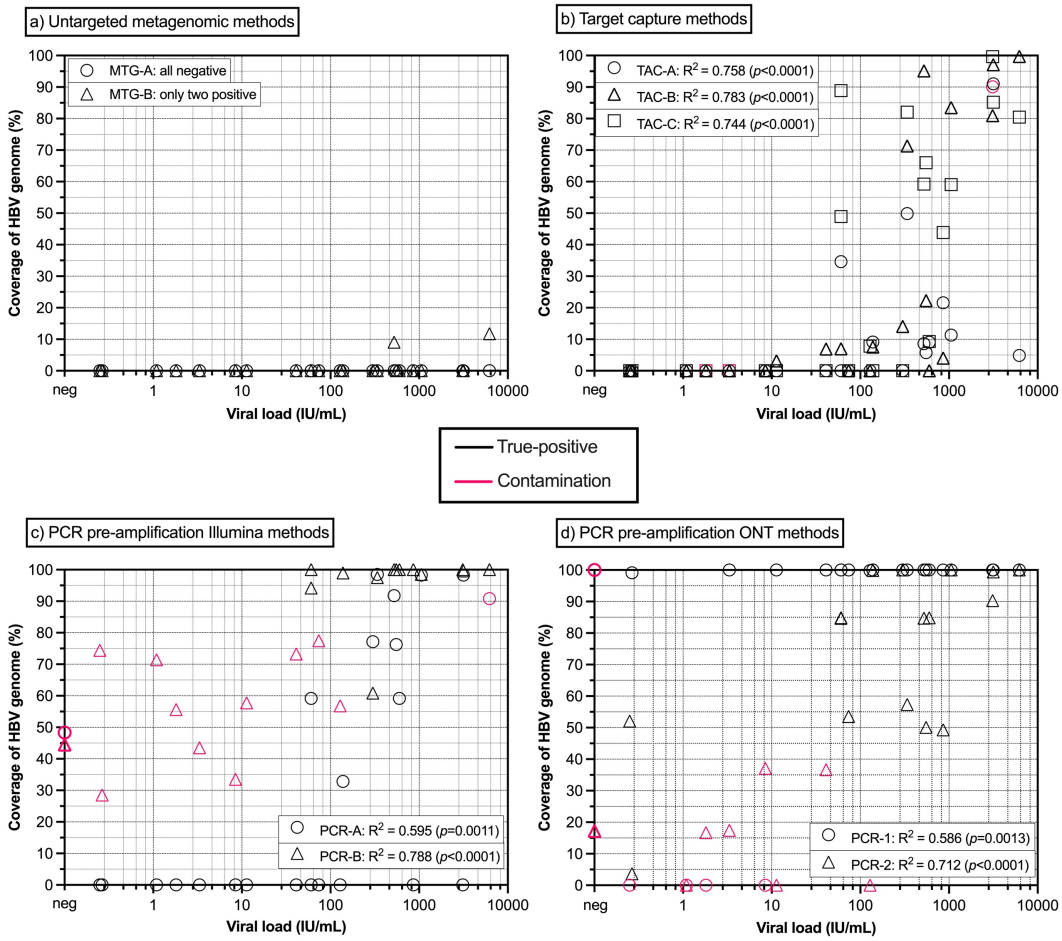
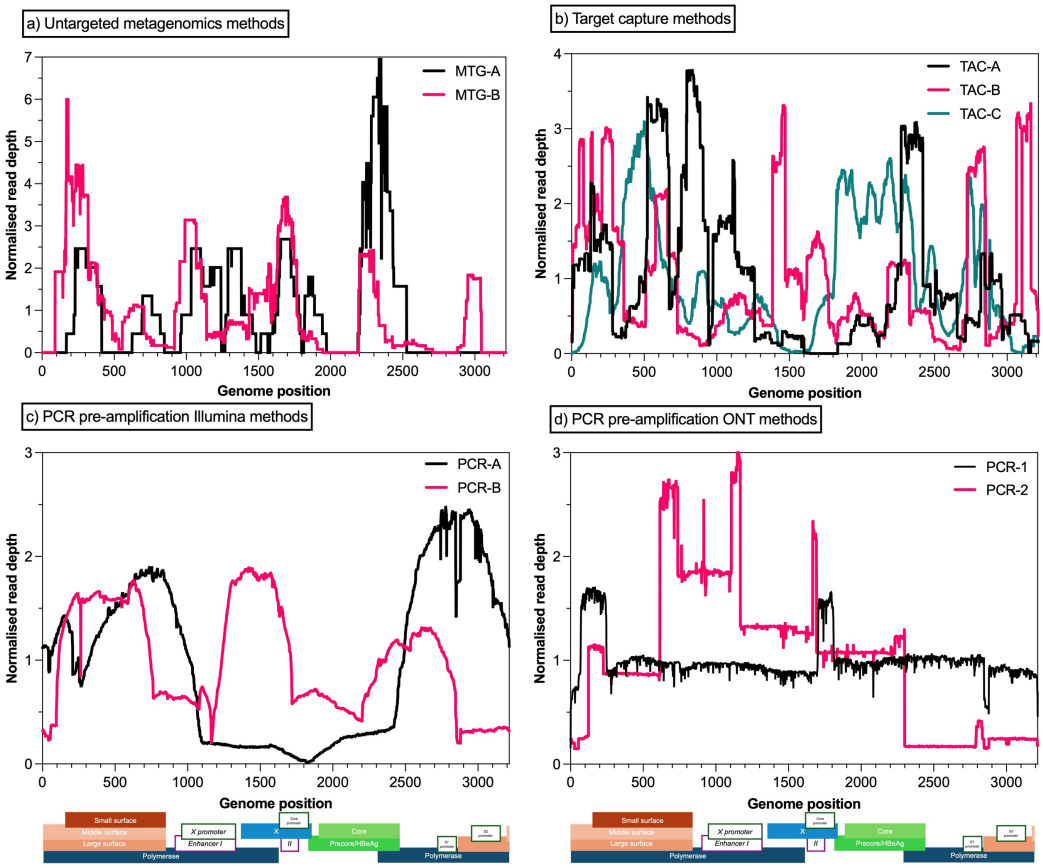


Figure 18B. Same as Figure 18A but applying a 10-base threshold.



Inspection of the mean genome coverage revealed that the methods yielded uneven coverage across the genome (Figure 19). Read depth was most uniform for PCR-ONT methods, while PCR-Illumina methods had more regions of reduced coverage. Coverage of the HBV genome was even sparser for the TAC and MTG methods. When analysing individual samples, there was considerable heterogeneity in the distribution of HBV genome coverage between protocols, without clear patterns.

Figure 19.[4] Normalised mean read depth of the HBV genome for each of the sequencing methods. The normalised mean read depth was calculated as the number of bases at each genome position as a proportion of the total reads of the sequence, then multiplied by the total number of sites, where the expected mean value was one base per site. The eleven highest viral load samples were included when detected, and only true-positive sequences with more than 1000 total HBV-specific bases read were included; the number of samples from each protocol that were included is indicated in each plot's key. Genome positions were based on the D00330 reference sequence. A genome diagram of HBV, drawn to the x-axis scale, shows gene positions in shaded boxes and regulatory regions in unshaded boxes. TAC: target capture; MTG: untargeted metagenomics.



Heterogeneity

The diversity of base counts at each site was quantified through calculations of Shannon entropy. Variability in Shannon values was observed between the NGS methods for the same samples, which may reflect the variations in the quantities of bases read by each method. MTG methods showed the least intra-sample HBV sequence heterogeneity, with a maximum mean Shannon entropy of 0.01, followed by the TAC methods (up to 0.04) and the PCR-Illumina methods (up to 0.09). However, heterogeneity was extremely high (up to 0.32) for PCR-ONT methods (Figure 20A); sub-analyses using a 10-base threshold for calling consensus bases did not affect these values (Figure 20B). The presence of ambiguous bases in sequences from PCR-ONT methods did not affect the perceived 95% accuracy of consensus sequences. Diversity and VL were poorly correlated for all methods, but diversity was lower when VL was higher for PCR-2 after applying a 10-base threshold.

Figure 20A.[4] Relationship between viral loads and mean Shannon entropy values for polymorphic sites for each group of methods on a common axes scale, when majority consensus bases were called at a threshold of 1 base per nucleotide site. Pink indicates contaminating sequences, including where HBV sequences were obtained for the negative HBV10 sample on the y-axis. Samples not detected are found on the x-axis. Spearman's correlation test deduced the significance of the association between viral loads and entropy values (R^2 value and *p*-values listed in boxes). HBV: hepatitis B virus; MTG: untargeted metagenomics; TAC: target capture; ONT: Oxford Nanopore Technologies.

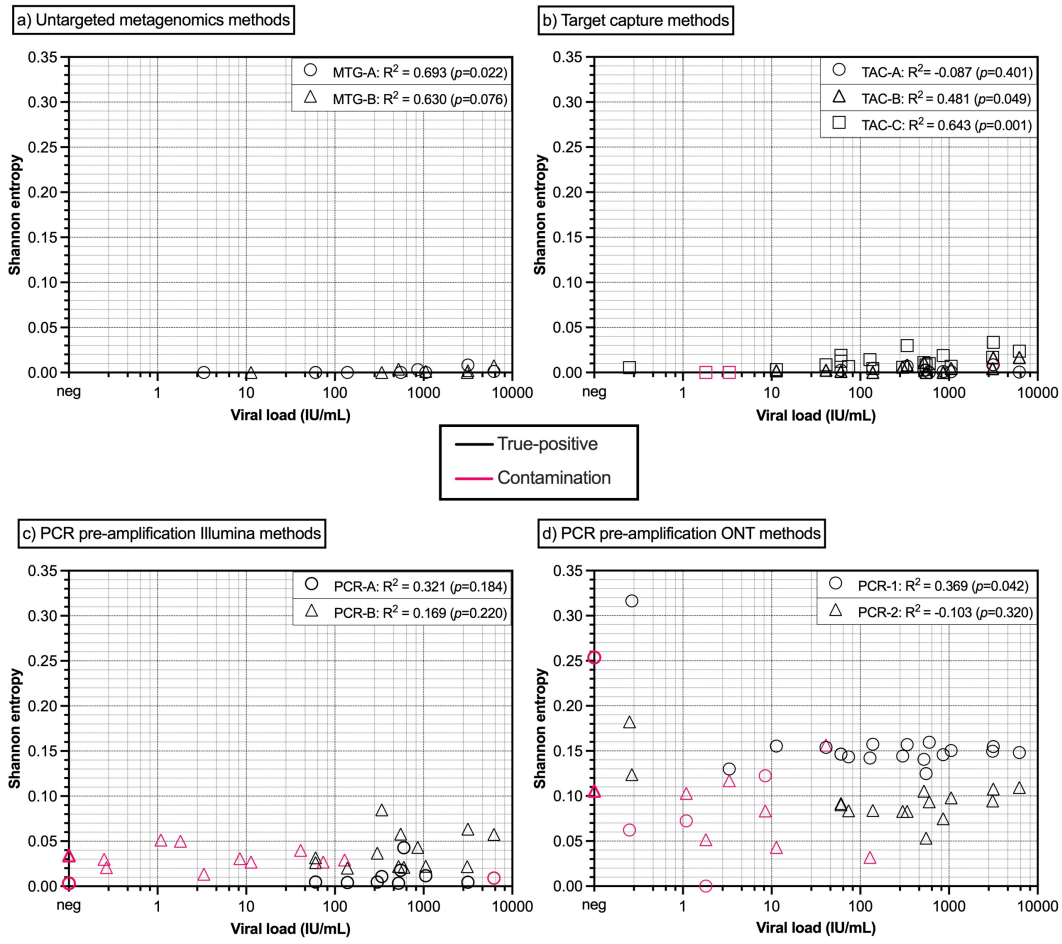
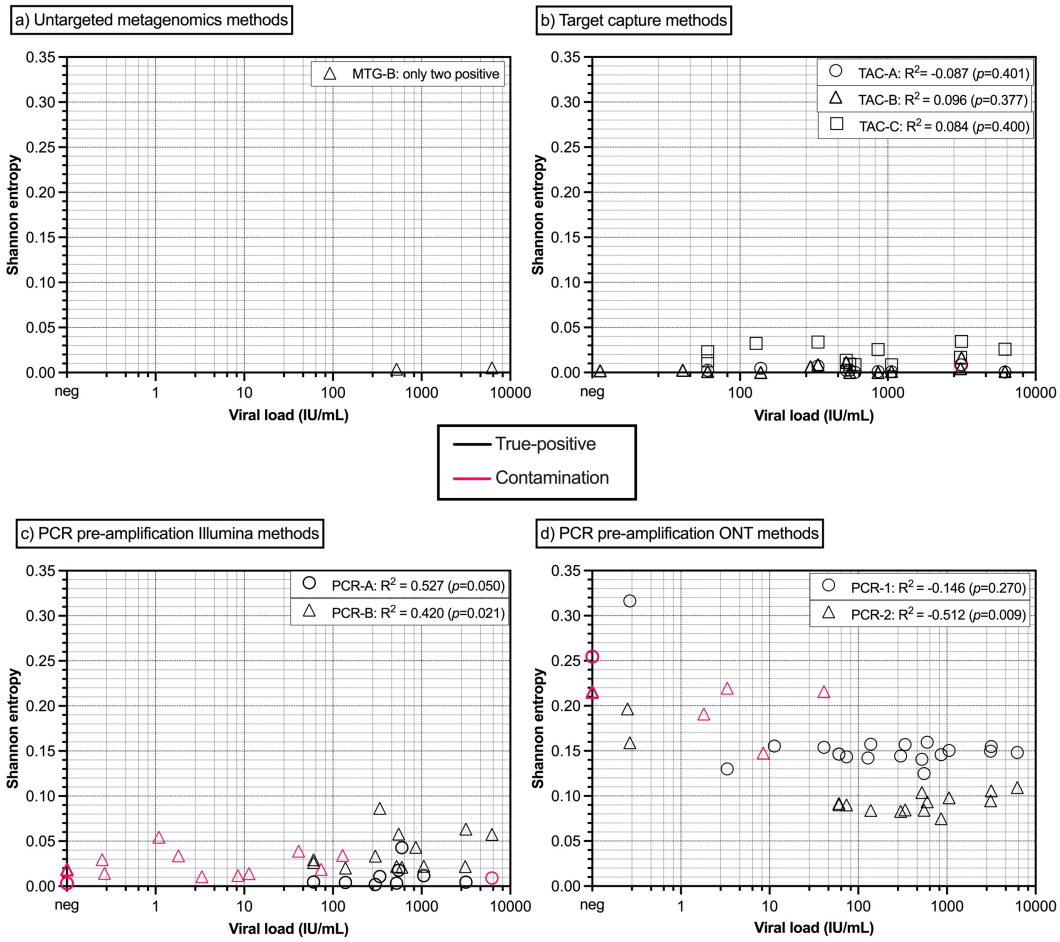


Figure 20B. Same as Figure 20A but applying a 10-base threshold.



Co-detection

Protocol-specific bioinformatic pipelines were employed to determine the presence, read counts, and genome coverage of other virus species for the TAC and MTG methods, which simultaneously screened for multiple viruses from generated sequences (Table 14). Altogether, anellovirus sequences were detected in 22/24 samples by the five NGS protocols. Anellovirus was detected by at least two protocols in 19/24 samples, and 2/24 samples by all five protocols. >50% genome coverage for anellovirus was observed in two samples. Confirmatory PCR for alpha, beta, and gammatorqueviruses revealed the presence of DNA sequences of all three anellovirus genera in all 24 samples (20/24 αTTV-positive αTTV; 22/24 βTTV-positive; 17/24 γTTV-positive), all with relatively high VLs (Ct values ranged from 4.7 to 26.1). Sanger sequencing of amplicons generated by nested PCR from randomly selected anellovirus-positive samples confirmed the presence of different strains from each genus (data not shown).

Table 14.[4] Detection of other viruses by the targeted capture and untargeted metagenomics methods, showing deduplicated read counts (genome coverage %). Results reported for TAC-C are for viruses where at least 10 reads were obtained. More than one TTV species may have been identified for MTG-A, but the most abundant was included. The furthest right column shows the PCR results, where PCR was performed for all samples when two or more methods detected a virus in the same sample. These PCRs were specific for alpha, beta and gamma-torquevirus, Merkel cell polyomavirus, and herpesviruses. '-' denotes where the method did not detect the virus, '=' denotes where the PCR was not performed. A numerical superscript indicates where the same laboratory performed the protocols. HHV: human herpesvirus; MCPyV: Merkel cell polyomavirus; AV: anellovirus; TTV: torquetenovirus; EBV: Epstein-Barr virus; KSHV: Kaposi's sarcoma-associated herpesvirus; JCPyV: John Cunningham virus.

Sample	Virus	MTG-A	MTG-B[1]	TAC-A[1]	TAC-B[1]	TAC-C	PCR
HBV1	AV	43 (54%)	-	6 (14%)	3 (3%)	37 (22%)	**Ct 13.1 (αTTV)**
	HHV7	-	-	-	-	885 (10%)	**Negative**
HBV2	MCPyV	-	-	-	-	14 (20%)	**Negative**
	AV	-	-	-	-	35 (21%)	**Ct 9.6 (αTTV), Ct 8.0 (βTTV), Ct 21.1 (γTTV)**
	HHV7	-	-	-	-	649 (10%)	**Negative**
HBV3	AV	9 (20%)	2 (1%)	7 (20%)	19 (12%)	167 (49%)	**Ct 6.3 (αTTV), Ct 5.8 (βTTV), Ct 8.5 (γTTV)**

	EBV	-	-	-	-	321 (6%)	**Negative**
	HHV7	-	-	-	-	1699 (9%)	**Negative**
HBV4	AV	26 (49%)	-	11 (20%)	14 (16%)	117 (45%)	**Ct 10.9 (αTTV), Ct 4.7 (βTTV), Ct 13.0 (γTTV)**
	Simian adenovirus 25	7 (0.74%)	-	-	-	-	=
HBV5	AV	-	5 (8%)	-	20 (16%)	15 (15%)	**Ct 22.2 (αTTV), Ct 11.0 (βTTV)**
	JCPyV	-	-	-	-	63 (52%)	=
HBV6	AV	6 (21%)	44 (40%)	7 (12%)	310 (75%)	84 (36%)	**Ct 7.2 (βTTV)**
	MCPyV	-	-	-	-	126 (65%)	**152 copies/mL**
	HPyV6	-	-	-	4 (4%)	26 (30%)	**Negative**
	HHV7	-	-	-	-	565 (10%)	**Negative**
	JCPyV	-	-	-	-	70 (56%)	=
HBV7	AV	-	2 (2%)	13 (6%)	11 (24%)	14 (17%)	**Ct 7.2 (γTTV)**
	HPV-2	-	-	-	-	10 (8%)	=
	HHV7	-	-	-	-	1112 (9%)	**Negative**
	JCPyV	-	-	-	-	34 (41%)	=
HBV8	EBV	12 (0.2%)	-	42 (14%)	3 (2%)	2020 (39%)	**Ct 35.9**
	AV	-	1 (1%)	1 (5%)	-	-	**Ct 12.5 (αTTV), Ct 8.3 (βTTV), Ct 21.4 (γTTV)**
	MCPyV	-	-	-	-	35 (31%)	**Negative**

HBV9	AV	-	-	3 (4%)	1 (1%)	-	**Ct 15.0 (αTTV), Ct 8.9 (βTTV), Ct 19.2 (γTTV)**
	HHV6	-	-	-	1 (1%)	1626 (15%)	**Ct 38.2**
HBV10	AV	-	3 (1%)	3 (4%)	1 (9%)	-	**Ct 12.6 (αTTV), Ct 10.7 (βTTV)**
	HHV7	-	-	-	-	730 (7%)	**Negative**
HBV11	AV	-	-	1 (6%)	2 (13%)	22 (10%)	**Ct 6.1 (αTTV), Ct 5.9 (βTTV), Ct 22.5 (γTTV)**
	EBV	-	-	-	-	774 (7%)	**Negative**
	HHV7	-	-	-	-	805 (7%)	**Negative**
HBV12	KSHV	18 (0.63%)	-	1 (5%)	-	7353 (93%)	**Ct 36.6**
	EBV	-	-	-	1 (1%)	-	**Negative**
	HHV7	-	-	-	-	1324 (9%)	**Negative**
	AV	-	2 (1%)	-	-	-	**Ct 26.1 (αTTV), Ct 13.3 (βTTV)**
HBV13	MCPyV	-	-	-	-	16 (15%)	**Negative**
	TTV	-	3 (2%)	-	-	20 (24%)	**Ct 11.9 (αTTV), Ct 5.1 (βTTV), Ct 13.0 (γTTV)**
	HHV7	-	-	-	-	825 (11%)	**Negative**
HBV14	AV	-	8 (11%)	-	88 (33%)	-	**Ct 7.0 (αTTV), Ct 10.1 (βTTV)**
	JCPyV	-	-	-	-	10 (10%)	**=**

	JCPyV	-	-	-	-	69 (59%)	=
HBV15	AV	7 (9.6%)	7 (1%)	-	16 (35%)	11 (8%)	**Ct 12.4 (αTTV), Ct 11.4 (βTTV), Ct 15.2 (γTTV)**
	EBV	-	-	-	1 (1%)	-	**Negative**
HBV16	AV	-	1 (1%)	-	1 (12%)	-	**Ct 13.8 (αTTV), Ct 9.1 (βTTV)**
HBV17	HHV7	-	-	-	-	413 (9%)	**Negative**
	AV	-	-	-	-	-	**Ct 13.3 (βTTV), Ct 15.3 (γTTV)**
HBV18	AV	-	-	1 (3%)	1 (1%)	-	**Ct 12.9 (αTTV), Ct 11.4 (βTTV), Ct 21.0 (γTTV)**
HBV19	AV	-	-	-	-	-	**Ct 19.1 (αTTV), Ct 16.2 (βTTV), Ct 9.5 (γTTV)**
HBV20	AV	27 (41%)	-	8 (13%)	2 (8%)	25 (18%)	**Ct 12.2 (αTTV), Ct 11.5 (βTTV), Ct 6.9 (γTTV)**
HBV21	TTV	-	-	1 (4%)	3 (9%)	-	**Ct 11.9 (βTTV), Ct 9.4 (γTTV)**
	HPV	-	-	-	-	17 (15%)	=
	HHV7	-	-	-	-	833 (9%)	**Negative**
	MCPyV	-	-	-	-	-	**219 copies/mL**

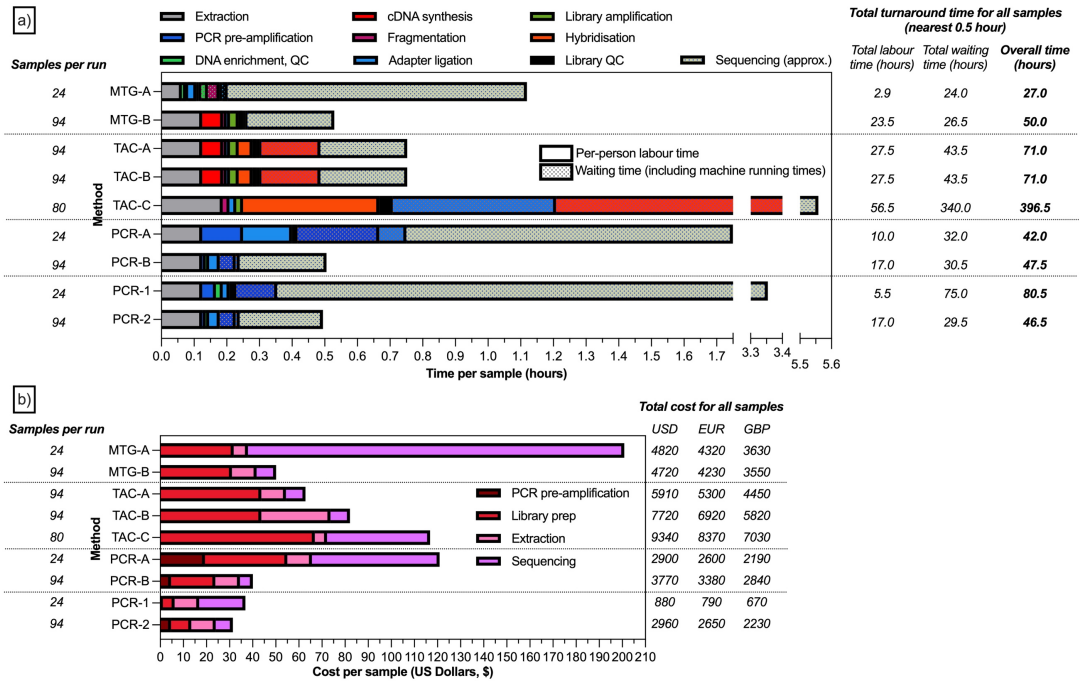
HBV22	AV	-	-	-	8 (11%)	-	**Ct 11.4 (αTTV), Ct 12.1 (βTTV), Ct 21.4 (γTTV)**
	HHV6	-	-	-	-	1070 (7%)	**Negative**
HBV23	AV	-	2 (1%)	11 (35%)	22 (26%)	56 (30%)	**Ct 5.8 (αTTV), Ct 6.4 (βTTV), Ct 19.8 (γTTV)**
	EBV	-	-	-	-	212 (6%)	**Negative**
	HHV6	-	-	-	-	1535 (7%)	**Negative**
	HHV7	-	-	-	-	761 (5%)	**Negative**
	MCPyV	-	-	-	-	10 (8%)	**Negative**
HBV24	AV	-	2 (1%)	22 (28%)	4 (6%)	31 (21%)	**Ct 7.8 (αTTV), Ct 7.0 (βTTV), Ct 13.5 (γTTV)**
	HHV6	-	-	-	-	616 (11%)	**Negative**
	HPyV7	-	-	-	-	13 (12%)	=
	HPyV6	-	-	-	-	27 (24%)	**Negative**
	KSHV	-	-	-	-	226 (5%)	**Negative**
	HHV7	-	-	-	-	326 (5%)	**Negative**

Various human herpesviruses were detected in 17/24 samples across the five MTG and TAC protocols at <50% genome coverage, and were detected by more than one protocol for three samples (EBV in 4/5 protocols in two laboratories for sample HBV8; HHV6 in 2/5 protocols in two laboratories for HBV9; KSHV in 3/5 protocols in three laboratories for HBV12). Real-time PCR confirmed the presence of these herpesviruses in these three samples, but no other sample was HHV PCR-positive, including the 13 samples where TAC-C reported HHV-7 DNA sequences. Polyomavirus DNA sequences were detected in 11/24 samples using NGS protocols; however, they were only detected by more than one protocol in one sample (HPyV6, detected by two protocols in two laboratories in HBV6). HyPV6 was not detected by real-time PCR. Instead, MCPyV was detected by real-time PCR in low copy numbers in HBV6 (detected by one NGS protocol) and HBV21 (not detected by any NGS protocol). Overall, TAC methods detected a greater number of viruses at higher read counts and coverage than MTG methods.

Time and costs

The per-person labour and waiting times varied between protocols (Figure 21A). The turnaround time was shortest for MTG and most PCR-based protocols. TAC methods took longer, mostly due to overnight hybridisation steps. Costs were lowest for PCR-ONT methods, higher for PCR-Illumina methods, followed by MTG methods, whilst TAC methods had the greatest cost (Figure 21B).

Figure 21.[4] Comparison of a) time taken, and b) cost based on the total time and costs for a protocol-specific representative number of samples included in a typical run. For Figure 5a, undotted bars to the left of the plot show per-person labour time per sample, whereas dotted bars to the right show the waiting time per sample, which may include machine running times. For clinical purposes, PCR-1 is normally run overnight on the MinIon flow cell (average of 16 hours), but was run for 72 hours for this study. Costs were standardised using a 1:0.8959:0.7531 USD:EUR:GBP exchange rate, as of May 18, 2025, and total costs were rounded to the nearest ten. USD: United States Dollar; EUR: Euro; GBP: Great British Pound; QC: quality control; TAC: target capture; MTG: untargeted metagenomics.



Discussion

This was the first multicentre comparison of currently available NGS methods in low HBV DNA VL clinical samples. Distinctive differences were found between the methods' capabilities in generating accurate HBV sequences at the lowest VLs, detecting other virus species, and their labour and monetary burden.

NGS methods involving pre-amplification steps reliably detected HBV at VL ~10 IU/ml (for PCR-1 and TAC-C) or ~100 IU/mL (for other pre-amplification protocols), where detection was often non-specific at the lowest VLs for PCR-based methods but was most costly for TAC methods. Previous investigations of PCR-1 and PCR-2 also showed successful amplification of full HBV genomes at VL <30 IU/ml[130,136], and alternative probe-capture methods observed a similar 50 copies/ml limit of detection for HBV[141] and 90-100% coverage for a range of DNA and RNA viruses at ≥1000 copies/ml[142,143]. The substantially reduced depth of coverage for MTG methods we observed has been well-described for a range of viruses[133–135]. However, the method retains its value in other applications due to its ability to detect unanticipated or potentially entirely novel pathogens without a requirement to pre-select targets in capture and PCR-assisted NGS methods[126].

Although detection sensitivity was similar, genomes generated from PCR-based methods were near-complete compared to lower genome coverage from TAC methods for our low VL samples. As an inexpensive method, PCR-ONT may be a beneficial screening tool for the HBV and microbiology fields, especially in low- and middle-income countries[144] when ONT allows preliminary analysis before a run is completed, which would be advantageous for high VL samples. Our findings contrast

with a similar study on hepatitis C virus, which concluded that PCR-based NGS was relatively laborious[135]; improvements in technologies and workflows could explain differences found. However, PCR-based NGS would be more challenging for more divergent and/or larger viruses than HBV, as primer design for whole-genome amplification can be difficult, and amplicon drop-out necessitating primer re-design is not uncommon. Variables that may have affected methods' detection capabilities in this study include extraction from larger volumes of plasma (as previously found with PCR-1[136] and TAC-A/B protocols[3]), having greater sample representation and fewer samples for library preparation, and sequencing more gigabases per sample. For PCR-based methods, sensitivity is affected less by the sequencing platform and relies heavily on the specific PCR approach, particularly the conservation of primer-binding sites. A nested PCR strategy with shorter amplicons would improve sensitivity if WGS is not required.

The frequent detection of HBV contaminants regardless of the presence or absence of the virus demonstrates a substantial limitation to the diagnostic reliability of PCR-based methods. Without other sequence data for comparison, minimum VL or base count thresholds need to be applied to detect and characterise sequences confidently. Alternatively, utilising a minimum threshold of 10 for calling consensus bases was useful for certain protocols to exclude background contamination (yet HBV was still detected in the negative control), and other protocols required additional genome coverage thresholds. The detection of contaminating sequences has been observed in other studies comparing NGS protocols[134,142], reinforcing this challenge for NGS sequencing in clinical diagnostic settings. For detecting and quantifying samples with very low VL, such as those infected with occult HBV[2], conventional

real-time or nested PCR would still be necessary. As with the detection of viruses around the limit of detection of conventional PCR assays, the stochastic effect around the limits of detection of NGS protocols affects the detection of samples with low VL, albeit at a higher detection limit than conventional PCR.

The high diversity of base counts and error rates for ONT base-calling algorithms hinders the detection of minority variants. Although these errors are unlikely to impact interpretation for most clinical and public health purposes, we advise against performing variant calling directly from mapped ONT reads without considerable bioinformatics expertise to exclude false substitutions and improve variant calling[145]. However, the introduction of R10 ONT kits and improved base-calling capabilities after our ONT protocols were performed has since changed the ONT field and may lower the diversity of base counts found in this chapter[146,147].

NGS methods involving PCR amplification are inherently limited to the characterisation of one known target pathogen and the length of that pathogen's genome; metagenomic methods showed clear advantages. Co-detections found high prevalences of anelloviruses, human herpesviruses, and polyomaviruses in the samples. Whilst the clinical relevance of these viruses for the healthy population and blood donation is questionable, the viruses may potentially cause harm if transmitted to immunocompromised individuals.

The 100% (24/24) prevalence of anellovirus DNA detection by PCR was consistent with previous studies, which have shown that most immunocompetent individuals carry the virus in their blood and at high VLs in patients with HBV

infection[148]. The low detection rate by all TAC protocols may have originated from the extreme genetic heterogeneity of the anellovirus species and genera that were not adequately represented among the probes used for target capture[149]. However, this does not explain the similarly low rates of detection by MTG methods which were not a sensitivity problem as the anelloviruses had high VLs by PCR; it is possible that their circular sequences were too divergent to map to what might be a very limited number of reference sequences used by the bioinformatic software to assign sequence reads. Our detection rates of polyomaviruses and HHVs were comparable to those reported in population studies[150,151]. The high detection rate of HHVs and polyomaviruses with TAC-C may be explained by DNAemia from disintegrating persistently infected cells[152], or by free fragmented DNA not detected by amplicon-specific real-time PCR.

This investigation only included samples with lower VL; sequencing samples with higher VL would have increased the proportion of samples with high read depths and complete genome coverage. Protocols using commercially available HBV-exclusive capture panels were not included, and host transcriptomic information was not investigated, which could be combined with pathogen metagenomics to improve accuracy[153]. Although much of the bioinformatics was centralised to facilitate comparison, the initial pipeline-specific steps not centrally performed could have affected sensitivity, such as the choice of taxonomic classifier[133]. Further follow-up investigation is planned to evaluate the performance of different bioinformatic pipelines to ascertain optimal tools for HBV characterisation. It is noted that pipelines have specific criteria for calling majority consensus bases and for detection (Table 15), outside of the criteria already used for read quality in this investigation.

Table 15.[4] Specific criteria used by protocols to call majority bases and positive detection.

Protocol	Minimum depth for majority base calls	Other quality filters for bases	Other criteria for positive detection
MTG-A	1	Mean base quality of 13	-
MTG-B, TAC-A, TAC-B	3	Map quality of 1.0 (Phred)	Minimum genome coverage 10%
TAC-C	1	-	-
PCR-A	80	-	-
PCR-B	5	-	-
PCR-1	10	-	Minimum amplicon threshold of 100 for resistance mutation assessment in the RT or for genotyping 10 times more reads in the sample than in the negative control
PCR-2	20	-	-

By comprehensively comparing four NGS sequencing methodologies for detecting and generating HBV genomes in low VL samples, HBV-specific PCR-amplification methods using ONT platforms were the most cost-efficient in detecting the most HBV-positive samples with the highest genome coverage. The assimilation of these protocols could facilitate the sensitive and rapid generation of global HBV whole genome sequence data. It could also facilitate in-depth investigation of OBI-specific mutations across the HBV genome; preliminary results from the three OBI samples included in the sample panel (HBV18, HBV20, and HBV24; the global consensus sequence from HBV3 was insufficient to perform mutation analysis) corroborate existing literature on the presence of immune-escape mutations as well as identifying further potential mutations that could be verified by larger-scale studies (Table 16). However, data shows that alternative methods to real-time PCR are not as sensitive in detecting low viral loads, complicated by the issue of contamination in complex NGS workflows. Minimisation of contamination necessitates experience, laboratory skills, and precautionary steps[154], such as using separate PCR tubes, exercising extreme care when pipetting and handling tubes, and including appropriate negative plasma controls. For samples with VL outside of the typical VL range observed in OBI, NGS could be a valuable screening tool in blood donations to obtain more detailed genetic information on HBV and other pathogens present in the donation, dependent on the purpose and need of such information. Nonetheless, for blood donor screening to prevent transfusion-transmitted infections from donors with OBI, the current two chapters have shown that currently available molecular methods to detect HBV DNA lack sensitivity or scalability. In order to ensure a safe blood supply, anti-HBc screening remains desirable to exclude all potentially infectious donations that may contain HBV DNA at low and fluctuating levels.

Table 16. Overview of the mutations observed in global consensus sequences obtained by NGS for OBI samples in the sample panel. Where mutations were observed in OBI in previous studies, these are referenced. SHB = small surface region; RT = reverse transcriptase domain. Mutations were identified through the geno2pheno software.

Sample	**SHB mutations**	**RT mutations**
HBV18	S45A[155] Q129P[156] S143T[155]	I53S D83I S106A N112H N124H M129L S135A W153R V163I
HBV20	T127P[155] S193L[157] L209V[157]	Y135S L217R N248H M309K
HBV24	W74C Q101R[158–160] L127S	D83L D135F

Chapter 3: False positive results in anti-HBc screening and impact of universal screening on the blood supply

Most of this chapter is based on the published paper:

Fu MX, Ingram J, Roberts C, et al. Blood donation screening for hepatitis B virus core antibodies: The importance of confirmatory testing and initial implication for rare blood donor groups. Vox Sang. 2024 May;119(5):447-459. doi: 10.1111/vox.13608.

Introduction

HBV is transmissible by transfusion from blood donors who test negative for HBsAg but positive for anti-HBc[161,162]. Many of these HBsAg-negative donors were found to have detectable HBV DNA and, therefore, recognised as having OBI[17]. However, very low levels of HBV DNA are only intermittently detectable in donors' blood[163164], and as found in the previous two chapters, require highly sensitive testing that is not commercially available. Given the significant association of anti-HBc positivity with the presence of HBV DNA in the liver and the peripheral blood[165] and hence posing a risk of HBV transmission via blood transfusion, universal screening for anti-HBc has been implemented in many countries, including the UK[166]. However, transmission of HBV has been limited to anti-HBc positive donors with low levels of anti-HBs antibodies (<100 IU/L); hence, donors with higher anti-HBs levels can potentially be considered safe[40].

Identification of HBsAg-negative donors with potential long-term persistent HBV infections is further complicated by the limited specificity of anti-HBc assays used for screening, with up to 60% of initially reactive samples remaining

unconfirmed[167–170]. The existence of an IgM component in commercial anti-HBc total assays inherently restricts specificity further[171]. Specificity can be improved using robust confirmatory testing, including secondary anti-HBc assays or neutralisation tests that measure the blocking of anti-HBc reactivity by using a soluble recombinant HBV core antigen (rHBcAg)[172]. Other means to identify if borderline anti-HBc positivity is a result of non-specificity or low levels of circulating anti-HBc[173] include the measurement of anti-HBc avidity, where the strength of binding of anti-HBc antibodies is measured by an avidity index (AI), which increases between acute and chronic HBV infections[174].

There is particular concern that the exclusion and deferral of anti-HBc positive donors could negatively impact donor populations with rare blood groups associated with the absence of high-incidence red cell antigens. Indeed, it has been shown that most donors with OBI in the UK were born in areas of the world where HBV infection is endemic[39]. This is also where 55% of donors of Black ethnicities have the Ro blood subtype, a subtype only seen in 2% of all UK donors[175]. Rare blood phenotypes are in demand. The Ro subtype is frequently transfused to Ro patients with sickle cell disorder[175] to prevent stroke and crisis. Furthermore, the rare Rh type, rr, denotes a rare subtype of ABO group O Rh D-negative, which is frequently transfused in emergencies[176]. The full impact of excluding anti-HBc-positive donors on the supply of units with rare blood groups in the UK remains unknown.

This chapter firstly investigates the effectiveness of two different anti-HBc assays to determine true anti-HBc positivity by applying an anti-HBc blocking assay and anti-HBc avidity test in a cohort of 397 blood donors reactive in one anti-HBc

assay. The chapter then investigates the representation of rare blood groups in deferred anti-HBc positive donors with high levels of anti-HBs antibodies.

Materials and methods

Clinical specimens

Three hundred and ninety-seven EDTA plasma samples were obtained from NHSBT that had tested negative for HBsAg (Abbott Prism or Alinity S) and repeatedly reactive for anti-HBc (Architect, Abbott total anti-HBc II assay; S/CO ratio ≥1.0 being considered reactive) between 16/Jun/2022 to 13/Jan/2023. These samples had also been screened negative for HBV DNA in pools of 24 (Roche Cobas 6800; 95% LOD1.4 IU/mL; calculated 95% LOD 33.6 IU/mL in individual donor level when tested in pools of 24) and for anti-HBs (Abbott Architect). Samples with anti-HBs levels <100 IU/L were tested further at NHSBT using a second anti-HBc assay (Murex total anti-HBc, DiaSorin) in duplicate, for anti-HBe (Biomerieux Vidas), and for HBV DNA using individual NAT (Grifols Procleix Panther; 95% LOD 4.5 IU/mL). Anti-HBc positivity was defined as anti-HBc positive in the Architect and Murex assays and either anti-HBe or anti-HBs positive. In contrast, samples that were only anti-HBc positive in Architect and Murex but negative for other HBV markers were considered anti-HBc inconclusive. Architect-positive but Murex-negative were considered anti-HBc negative.

Clinical HBV testing at NHSBT was limited to samples with anti-HBs titres <100 IU/L as described above, but not on samples with higher anti-HBs titres. To harmonise testing for the entire sample set regardless of anti-HBs titre, we tested samples with anti-HBs titres >100 IU/L for HBV DNA individually (Roche Cobas 6800; 95% LOD 1.4 IU/mL; n=250). We then assayed all 397 samples by anti-HBc (Murex total anti-HBc, DiaSorin, Italy) and anti-HBe (FineTest Human HBeAb

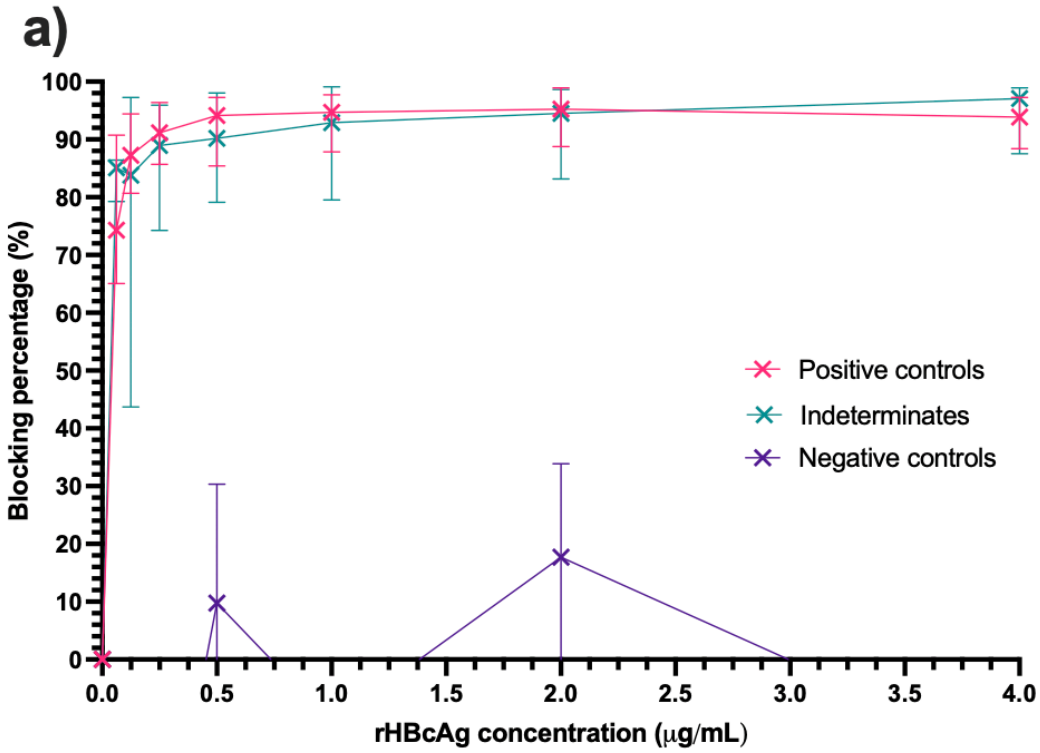
ELISA kit, Wuhan, China) testing following manufacturers' instructions. 13 random anti-HBs <100 IU/L samples, which were initially tested with the Murex assay at the clinical laboratory, were also tested at the research laboratory, where a correlation of similar anti-HBc S/CO ratios was found (r=0.940, p<0.001). Further, testing of 19 random anti-HBs <100 IU/L samples (consisting of ten anti-HBe negatives and nine anti-HBe positives) with the FineTest kit revealed 100% concordant qualitative results with the VIDAS assay, and quantitative values strongly correlated (r=0.794, p<0.001). The control panel of 26 HBV DNA-positive samples described in Chapter 1 were used as additional positive controls.

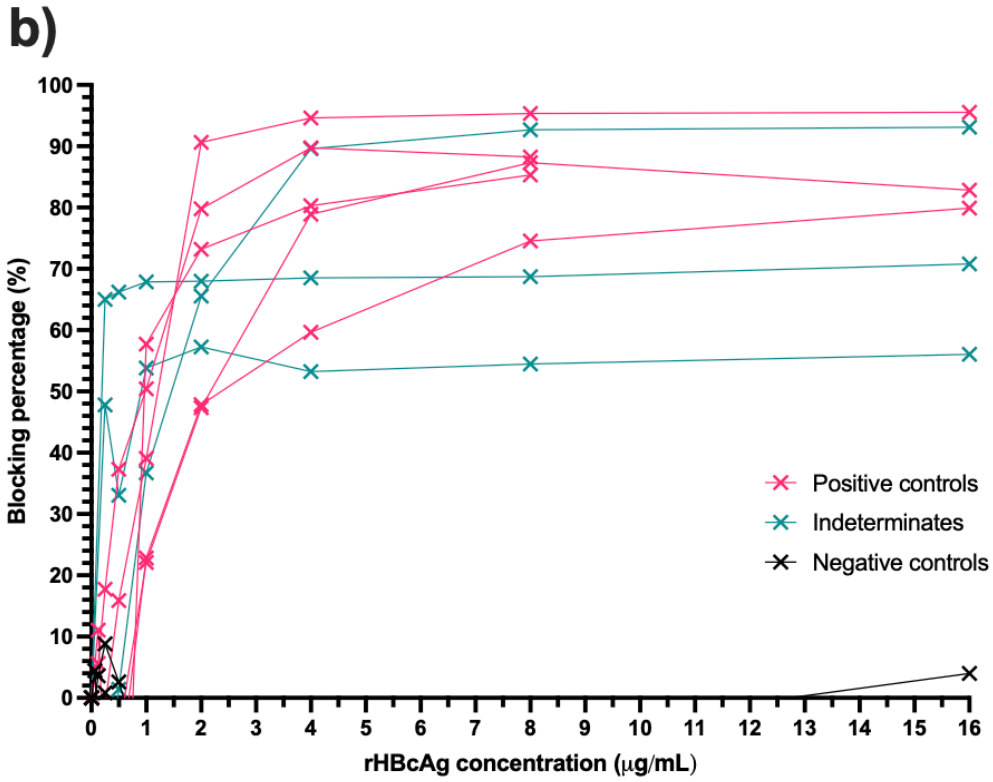
Demographic data were obtained from donors at the time of donation. The self-reported ethnicities of all 297,949 blood donors screened for anti-HBc in the one year since anti-HBc screening began in England from 30/June/2022 to 30/June/2023 were collated for comparison. The Blood Supply Clinical Audit, Risk and Effectiveness Research and Development Subcommittee of NHSBT approved this study. Two MSc projects contributed towards this study and were approved by the NHSBT Research and Development Committee. All methods followed the relevant guidelines and regulations under the Declaration of Helsinki. Informed consent was obtained at the time of donation.

In-house blocking assay

In-house non-blocking and blocking assays were developed to study anti-HBc immune responses. For blocking anti-HBc reactivity using rHBcAg, 50 μL of each antigen dilution was pre-incubated with 50 μL of plasma before plate washing and subsequent addition of IgG. If specific anti-HBc IgG antibodies were present, these would be neutralised by rHBcAg. For ELISA coating, serial dilutions of commercially available rHBcAg (Abcam ab49013, representing HBV320 strain of genotype D expressed in E. coli) were made from 4 g/mL to 0.025 g/mL. Comparisons of the optical density (OD) ratios of 1:100 dilutions of anti-HBc positive samples (anti-HBc positive in both screening assays) to anti-HBc negative samples (samples that tested anti-HBc negative in the second screening assay) found that the optimal coating concentration was 0.25 μg/mL. With a range of samples and varying rHBcAg concentrations from 0.0625 μg/mL to 4 μg/mL diluted in phosphate-buffered saline Tween (PBST) and 10 mg/mL milk powder, 0.25 μg/mL of rHBcAg was found to sufficiently inhibit ≥50% reactivity, whilst 1 μg/mL of rHBcAg maximally inhibited anti-HBc reactivity (Figure 22A). Subsequent sera incubation with rHBcAg was performed with 0.25 μg/mL and 1 μg/mL concentrations, where duplicates of sera with PBST and 10 mg/mL milk powder without rHBcAg competition were assayed as controls.

Figure 22.[5] Determination of the optimal concentrations of blocking recombinant HBV core antigen (rHBcAg) on the A) in-house anti-HBc IgG assay and the B) Murex anti-HBc assay. 1:2 serial dilutions of rHBcAg were used with anti-HBc positive samples (anti-HBc reactive in both screening assays, anti-HBe positive), initially indeterminate samples (anti-HBc reactive in both screening assays, anti-HBe negative), and negative controls (anti-HBc negative in the Murex assay, anti-HBe negative). The median [range] blocking percentages are displayed in plot a) for each category of samples, where rHBcAg concentrations of 0.25 μg/mL and 1 μg/mL block ≥50% anti-HBc reactivity and block the most signals, respectively. For plot a), twenty confirmed anti-HBc positive controls (comprising 17 HBsAg-positive samples from the HBV-positive control panel previously described[2] and three anti-HBe positive samples from the current study), thirteen anti-HBc indeterminates and five anti-HBc negative controls (comprising four anti-HBc initial reactive but negative in the second assay plus a negative pool of sera consisting of three donations tested negative for HBV markers) were used to develop the blocking assay. Plot b) displays the blocking percentages for each sample, determined using 1:2 serial dilutions of the commercial rHBcAg from 16 μg/mL to 0.25 μg/mL, together with a panel of 5 anti-HBc positive controls, three indeterminates, and three negative controls.


a)
Blocking percentage (%)
rHBcAg concentration (μg/mL)
Positive controls
Indeterminates
Negative controls


b)
Blocking percentage (%)
rHBcAg concentration (μg/mL)
Positive controls
Indeterminates
Negative controls

To ascertain the capacity of the commercial rHBcAg of HBV genotype D to block anti-HBc antibodies induced by various HBV genotypes, we tested 17 HBsAg-positive samples from an HBV control panel of genotypes A-E previously described[2], where efficient blocking of anti-HBc reactivity was shown across genotypes at the chosen concentrations (Figure 23).

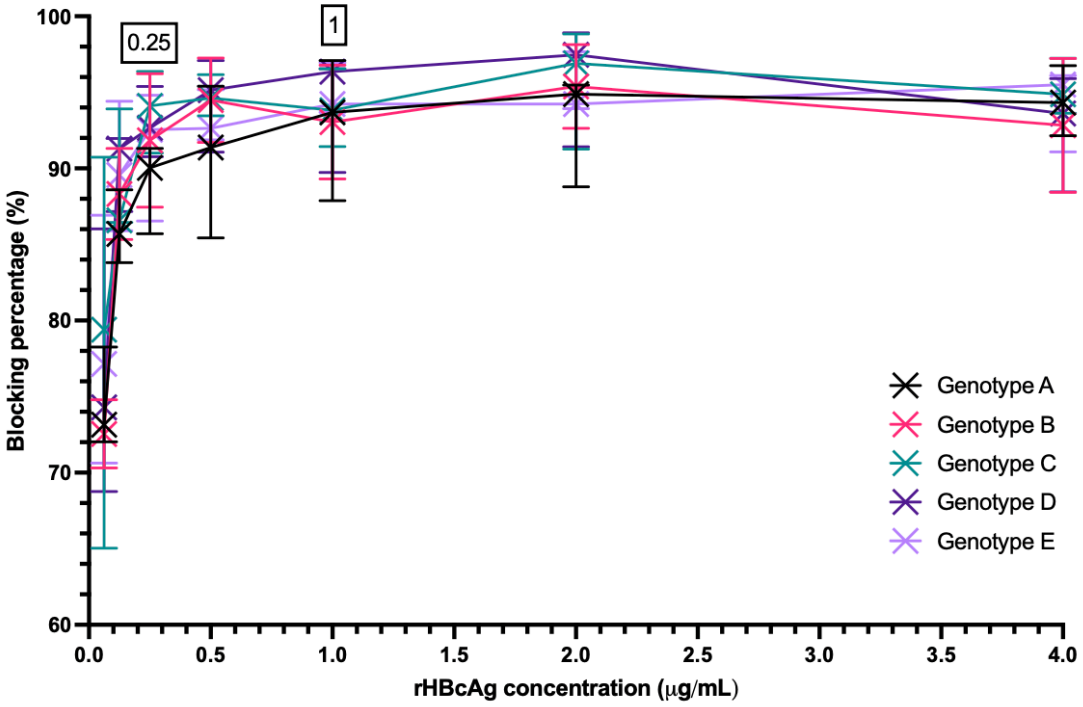


Figure 23.[5] Determination of the capacity of rHBcAg of genotype D to block anti-HBc antibodies induced by various HBV genotypes using samples from an HBV-positive control panel. Included were four samples of genotype A, two samples of genotype B, three samples of genotype C, five samples of genotype D, and three samples of genotype E. The median [range] blocking percentages of each genotype are displayed. The chosen blocking concentrations are written above the relevant points.

Standard curve and negative run controls

In each ELISA plate in the in-house assay, serial dilutions of the First International Standard for anti-HBc (National Institute for Biological Standards and Control, 95/522) were used for standard curve calibration of anti-HBc IgG results into IU/mL. The anti-HBc negative pool was also included in duplicate as negative controls on each plate. A separate set of ten anti-HBc negative controls was assayed to determine their mean and standard deviation (SD), where an OD threshold for anti-HBc positivity for each plate in the in-house assay was established by scaling the negative controls on each plate through the following equation:

$$OD\ cut\ off = Mean\ OD\ + \left\{\frac{Mean\ OD\ of\ plate\ controls}{Mean\ OD\ of\ 10\ controls} \times 2(SD\ of\ 10\ controls)\right\}$$

Murex blocking assay.

The blocking principle was applied to the anti-HBc Murex commercial assay, where undiluted sera were incubated with rHBcAg before initiating the manufacturer's protocol. A 2 μg/mL concentration of rHBcAg inhibited ≥50% reactivity sufficiently, and 8 μg/mL of rHBcAg inhibited anti-HBc reactivity maximally (Figure 22B). For higher-titre samples, higher concentrations of blocking rHBcAg were used. Samples in both blocking assays were considered blocked if both rHBcAg concentrations inhibited ≥50% anti-HBc reactivity. The blocking percentages were calculated as follows for the in-house blocking assay, with the numerator and denominator reversed for the competitive Murex blocking assay:

$$Blocking\ \% = 100 - \left\{\frac{Average\ OD\ of\ sera\ duplicate\ blocked\ by\ both\ rHBcAg\ concentrations}{Average\ OD\ of\ sera\ duplicate\ without\ blocking\ rHBcAg}\right\} x\ 100$$

Samples were diluted according to the screening Architect S/CO ratios, aiming for OD values between 1.0 and 2.0 to normalise the denominator for calculating blocking percentages.

In-house avidity assay

We adopted the avidity index (AI) method, initially developed for rubella serology[177], to assay anti-HBc avidity. The optimal urea concentration of 6M was determined with serial dilutions from 10M to 2M, ten HBV-positive control panel samples, and one acute high-titre IgM anti-HBc-positive HBV sample received from NHSBT (Figure 24). Serial 1:2 dilutions of nine chronic and one acute HBV samples were then assayed to ascertain the linear range of OD values producing consistent AI across sample dilutions (Figure 25); samples were subsequently diluted to obtain OD values between 0.700 and 2.000 in the wells without urea where the dilutions had minimal effect on the AI.

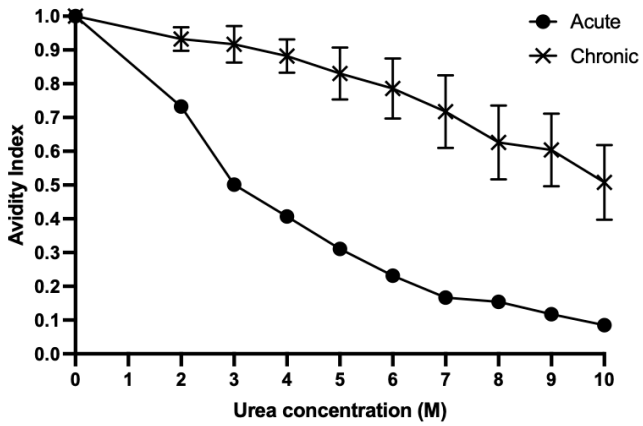


Figure 24.[5] Determination of the optimal urea concentration with serial dilutions of urea in phosphate-buffered saline Tween (PBST) from 10M to 2M and ten HBsAg-positive samples from the control panel and one IgM-positive sample. Error bars represent the means [standard deviations]. A concentration of 6M provided the highest difference in avidity between the chronic and acute HBV samples.

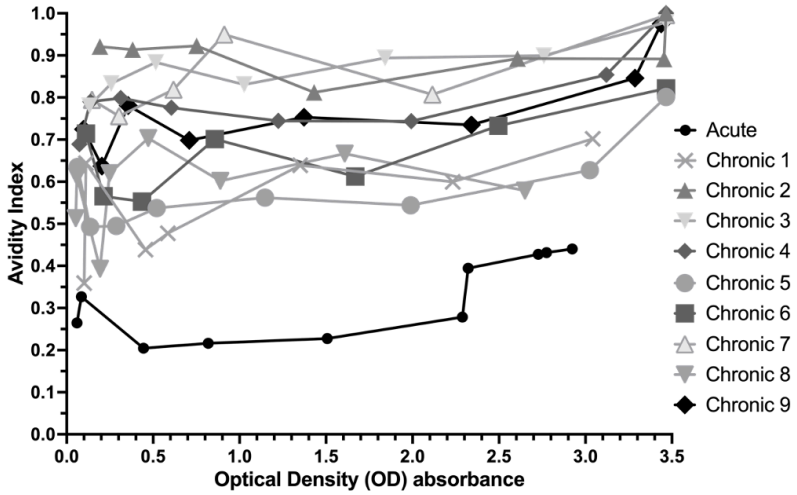


Figure 25.[5] Determination of the linear range of optical density (OD) absorbance values that produce consistent avidity indices between 1:2 dilutions of nine chronic and one acute HBV samples (where the data points at the far right represent the initial dilutions at the upper limit of detection, and each subsequent data point to the left represents sequential 1:2 dilutions). The avidity index for each sample at each dilution is shown.

Following the incubation of four aliquots of diluted sera in each rHBcAg-coated well and aspiration of the sera, two of the four wells that had serum aliquots were incubated three times for 5 minutes each with urea (which destabilises antibody binding) in PBST, and the two other wells that had serum aliquots with PBST only. The liquid in the wells was aspirated between each incubation with urea and PBST. All wells were then washed once before completing the ELISA. The AI for each sample was calculated as the average OD of wells incubated with urea divided by the average of wells without urea.

Rare donor analysis

Donors with rare blood types, alongside their ethnicities, were identified from the first anonymised 981 blood donors who were anti-HBc repeat reactive with anti-HBs ≥100 IU/L in the first six months of anti-HBc screening in England between 31/May/2022 and 30/November/2022. In the context of this chapter, rare blood types were defined as the following Rh genotypes: DcE/DcE (R2R2), Dce/dce (Ror, shortened to “Ro”) and dce/dce (rr). The number of positive anti-HBc and high titre anti-HBs donors of different ethnic groups within groups of rare Rh blood types was compared to the number of all active donors in the same ethnic groups from 30/June/2022 to 30/June/2023, excluding the 981 anti-HBc positive donors.

Statistical analyses

Data normality was assessed with Shapiro-Wilks tests, where normal data are displayed as mean ± SD and non-normal data as median [interquartile range] where applicable. Spearman's correlation compared assay results between research and diagnostic laboratories. Fisher's exact tests compared categorical variables. Mann-Whitney U or t-tests compared continuous variables between two groups, while the Kruskal-Willis test or one-way ANOVA compared continuous variables with more than two groups. ROC analysis investigated the sensitivities and specificities of anti-HBc Architect ratios and anti-HBe titres that may predict anti-HBc positivity. All analyses were performed with GraphPad Prism (v10.0.2, LLC). Statistical significance was set at $p \leq 0.05$.

Results

Validation of anti-HBc blocking assay

Validation of the assay was performed using 147 samples of known anti-HBc positive status (all reactive in two anti-HBc assays [Abbot Architect and Murex] and one anti-HBe assay [Murex or Finetest]) and 55 anti-HBc negative controls (weakly reactive in one anti-HBc assay [Abbott Architect] but negative in further anti-HBc assay [Murex] and for anti-HBs as well as for anti-HBe), selected from 397 study samples. In addition, 26 previously characterised HBV DNA-positive samples were included as further positive controls[2]. Architect anti-HBc ratios correlated better with in-house IU/mL values than Murex anti-HBc ratios (Figure 26).

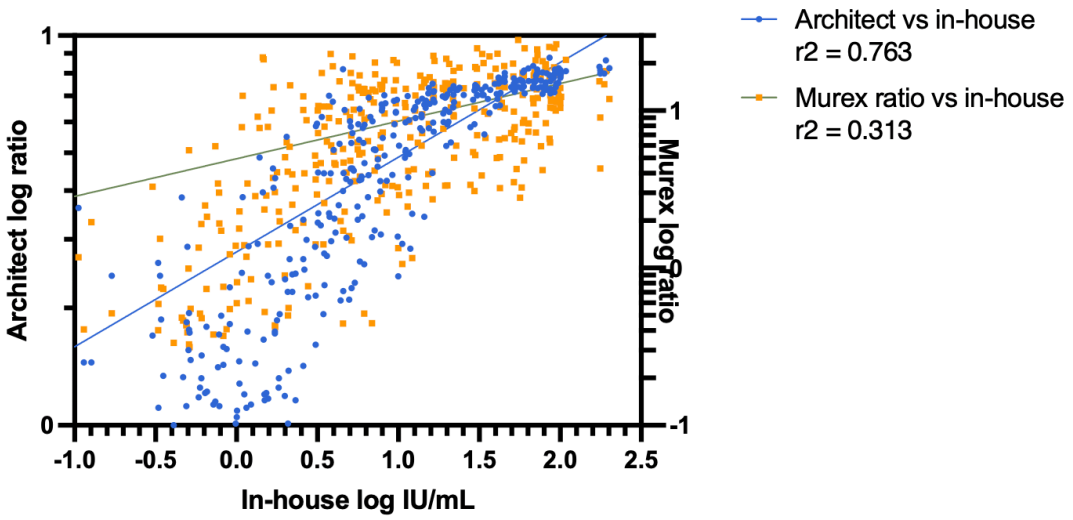
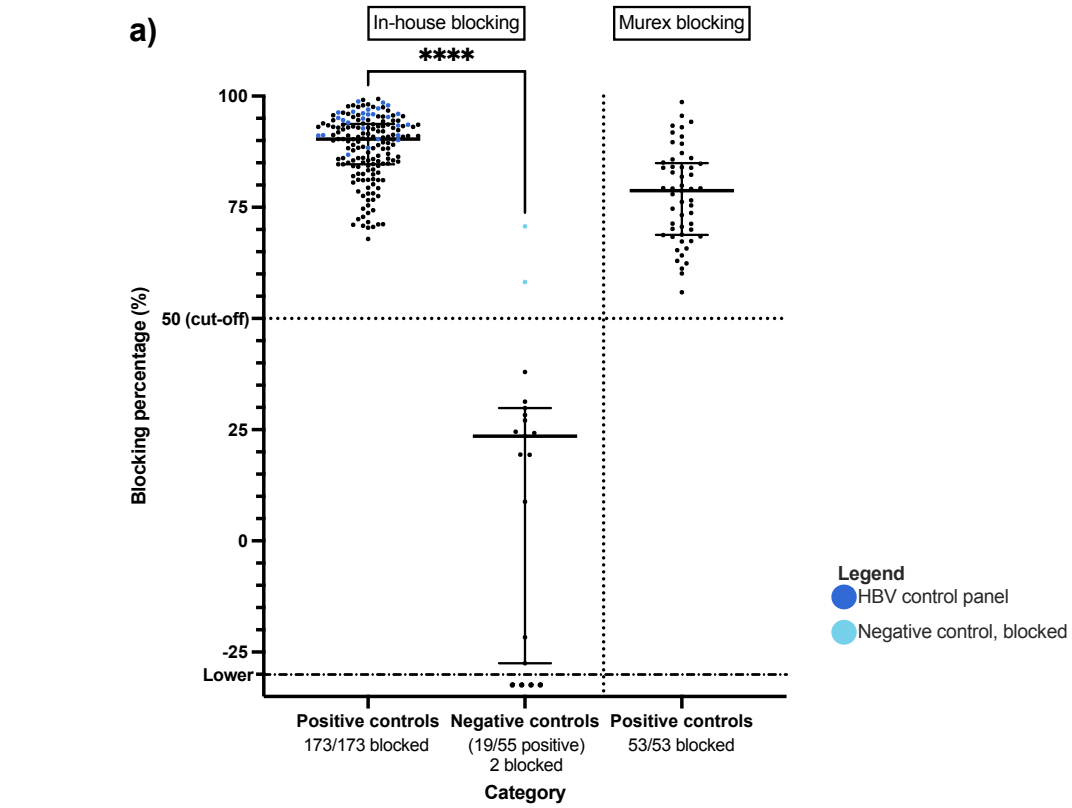
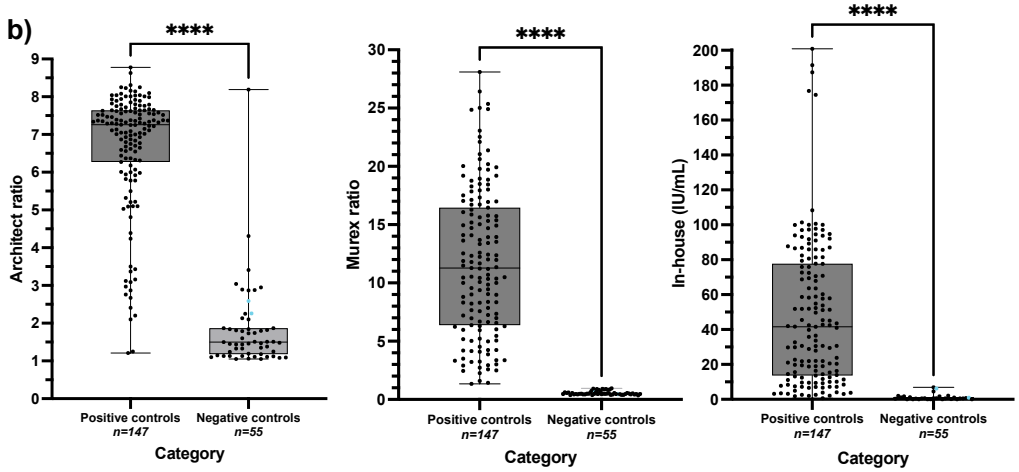


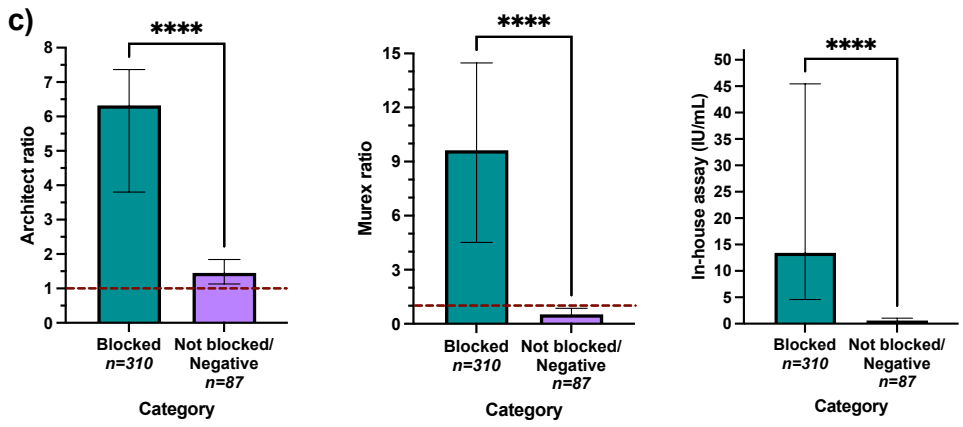
Figure 26.[5] Correlation of log IU/mL values of confirmed anti-HBc positive samples in the in-house assay with the Architect and Murex screening log ratios.

All anti-HBe-positive and HBV DNA-positive positive control samples were blocked by soluble rHBcAg in the HBcrAg ELISA based on a greater than 50% reduction of anti-HBc binding (range 69% - 94%; n=173; Figure 27A). Architect, Murex, and in-house anti-HBc titres were significantly higher (p<0.0001) in the positive than in the negative controls (Figure 27B). However, two anti-HBc negative controls were also blocked, with anti-HBc titres higher than the third quartile above the negative control mean (Figure 27). The remaining samples reactive in the Architect anti-HBc assay (n=195) were then tested for blocking. Combining the results, significantly higher anti-HBc titres in Murex, Architect, and rHBcAg ELISA assays were observed in blocked samples than unblocked or unreactive in the rHBcAg ELISA (Figure 27C; n=397).

Figure 27.[5] a) Median [interquartile range] blocking percentages in all 173 anti-HBc positive controls (147 anti-HBc reactive in both screening assays, anti-HBe positive and 26 samples from the HBV control panel) and anti-HBc negative controls (anti-HBc negative in the second screening assay, anti-HBe negative, anti-HBs negative) tested in the in-house assay. Thirty-two of 51 negative controls were anti-HBc negative in the in-house assay, and the other 19 controls underwent blocking. The blocking percentages of 23 anti-HBc positive controls tested in the Murex assay are also shown; b) Comparison of Architect, Murex and in-house anti-HBc ratios between positive and negative controls, showing all quartiles; c) Comparisons of the median [interquartile range] Architect, Murex and in-house anti-HBc ratios between donor samples that were anti-HBc blocked (n=310) and samples that were either not blocked or were anti-HBc negative (n=87). The dotted lines represent the 1.0 cut-off value for the anti-HBc screening assays. **** indicates $p<0.0001$ from Mann-Whitney U tests.


a)
In-house blocking
Murex blocking
****
100
75
50 (cut-off)
25
0
-25
Lower
Blocking percentage (%)
Positive controls
173/173 blocked
Negative controls
(19/55 positive)
2 blocked
Positive controls
53/53 blocked
Category
Legend
HBV control panel
Negative control, blocked

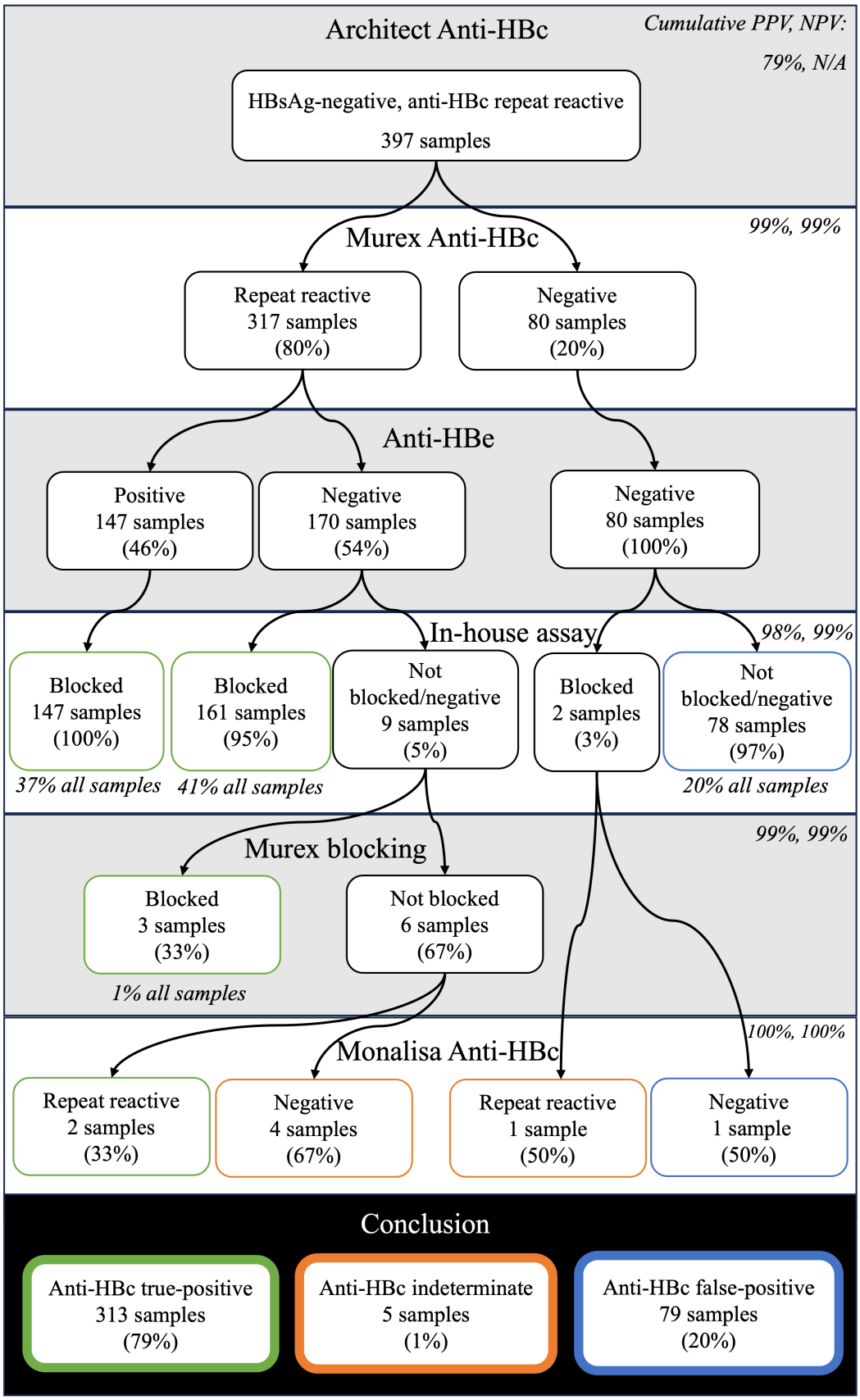

b)
****
Architect ratio
Murex ratio
In-house (IU/mL)
Positive controls
n=147
Negative controls
n=55
Category


c)
****
Architect ratio
Murex ratio
In-house assay (IU/mL)
Blocked
n=310
Not blocked/
Negative
n=87
Category

Serological correlates of confirmed anti-HBc positivity

A total of 317 of 397 anti-HBc initial Architect-reactive samples were positive in the Murex assay (80%). Of these, reactivity in the rHBcAg ELISA was blocked by soluble rHBcAg in 308 samples (308/317; 97%). However, reactivity was also blocked in two Architect-positive, Murex-negative, non-confirmed, presumed anti-HBc negative samples (2/80, 2,5%; Figure 28). The neutralisation observed in the latter two samples could not be blocked using a cell lysate from the strain DH5α of *E. coli* with a comparable GCE33-YFP expression plasmid without rHBcAg, ruling out non-specificity from that source.

Figure 28.[5] The flow diagram shows the delineation of anti-HBc reactivity based on extensive testing of the 397 HBsAg-negative and anti-HBc initial reactive samples on the Architect assay. Testing included the Murex anti-HBc assay, anti-HBe testing, in-house anti-HBc blocking assay, blocking on the Murex assay, and the anti-HBc LIASION test. The cumulative positive predictive value (PPV) and negative predictive value (NPV) of the five anti-HBc assays to the true-positive and false-positive samples in the 'Conclusion' are shown in the top-right of each anti-HBc assay, where minimal changes are observed after the Murex assay.

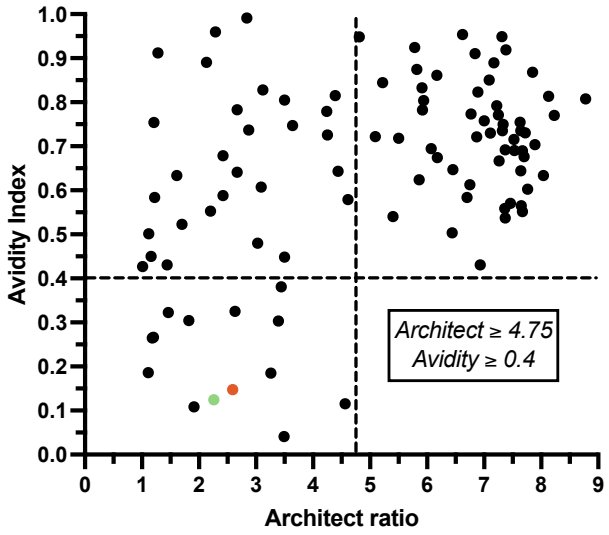

Architect Anti-HBc
Cumulative PPV, NPV:
79%, N/A
HBsAg-negative, anti-HBc repeat reactive
397 samples
99%, 99%
Murex Anti-HBc
Repeat reactive
317 samples
(80%)
Negative
80 samples
(20%)
Anti-HBe
Positive
147 samples
(46%)
Negative
170 samples
(54%)
Negative
80 samples
(100%)
In-house assay
98%, 99%
Blocked
147 samples
(100%)
Blocked
161 samples
(95%)
Not
blocked/negative
9 samples
(5%)
Blocked
2 samples
(3%)
Not
blocked/negative
78 samples
(97%)
37% all samples
41% all samples
20% all samples
99%, 99%
Murex blocking
Blocked
3 samples
(33%)
Not blocked
6 samples
(67%)
1% all samples
100%, 100%
Monalisa Anti-HBc
Repeat reactive
2 samples
(33%)
Negative
4 samples
(67%)
Repeat reactive
1 sample
(50%)
Negative
1 sample
(50%)
Conclusion
Anti-HBc true-positive
313 samples
(79%)
Anti-HBc indeterminate
5 samples
(1%)
Anti-HBc false-positive
79 samples
(20%)

Anti-HBc avidity

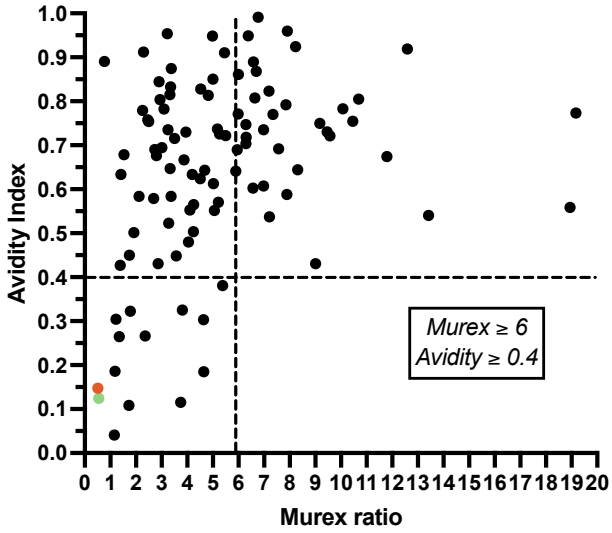
Ninety-nine samples positive in the in-house anti-HBc assay were tested to investigate the relationship between anti-HBc avidity (AI) and anti-HBc reactivity. Reactivity in anti-HBc assays was highly predictive of avidity (Figure 29): anti-HBc ratios of ≥4.75 and ≥6 in the Architect and Murex assays, respectively, predicted AI values of >0.4 and excluded lower AI and anti-HBc titres ≥14 IU/mL in the in-house assay predicted AI values of >0.5 and excluded lower AI. When assessing the two discrepant Architect-positive but Murex-negative samples blocked in the in-house blocking assay, both had similarly low avidities irrespective of positivity in the Monalisa assay.

Figure 29.[5] Correlation between avidity and anti-HBc a) Architect S/CO ratio (r^2=0.196), b) anti-HBc Murex S/CO ratio (r^2=0.109), and c) anti-HBc IU/mL from in-house assay (r^2=0.006), for all 99 samples tested for avidity and blocked. The dashed lines separate each anti-HBc ratio into a cut-off value for samples with lower avidity.

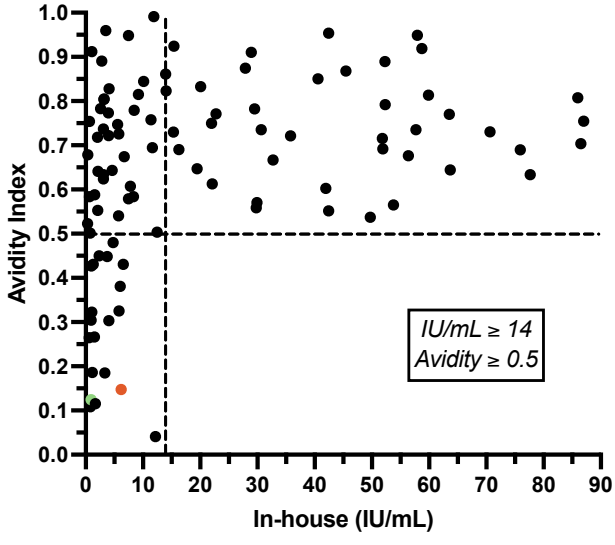
a)

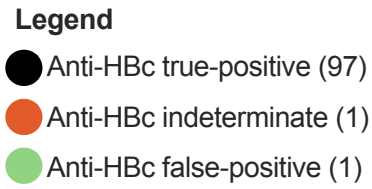

Avidity Index
Architect ratio
Architect ≥ 4.75
Avidity ≥ 0.4

b)


Avidity Index
Murex ratio
Murex ≥ 6
Avidity ≥ 0.4

c)


Avidity Index
In-house (IU/mL)
IU/mL ≥ 14
Avidity ≥ 0.5


Legend
Anti-HBc true-positive (97)
Anti-HBc indeterminate (1)
Anti-HBc false-positive (1)

Overall, five (1%) anti-HBc initially Architect-reactive samples remained anti-HBc indeterminate after extensive confirmatory testing (Figure 28; Table 17). 79% of anti-HBc initial Architect-reactive samples were genuine anti-HBc positive, whilst 20% were confirmed to be false-positive. 98% (166 of 170) of isolated anti-HBc positive samples concurrently Architect and Murex positive without anti-HBe antibodies were shown to be genuinely anti-HBc positive.

Sample	Architect (ratio)	Murex (ratio)	In-House Blocking (%)	Murex Blocking (%)	Monalisa (ratio)	Anti-HBe (ratio)	Anti-HBs (ratio)*	Avidity Index	Ethnicity
Pos-1	1.15	5.94	NA	73	1.132	0.55	>1000	NA	White British/Irish
Pos-2	1.20	2.35	24	68	2.256	0.55	>1000	0.266	Other Ethnic Groups
Pos-3	1.72	3.23	NA	87	1.178	0.62	0.00	NA	White British/Irish
Pos-4	1.70	3.28	-44	6	1.938	0.52	462	0.523	White British/Irish
Pos-5	2.42	1.53	21	38	2.45	0.39	2.18	0.678	White British/Irish
Ind-1	1.29	2.24	NA	16	0.709	0.00	0.29	NA	White British/Irish
Ind-2	1.32	1.91	NA	47	0.946	0.00	1.52	NA	White British/Irish
Ind-3	1.53	1.23	NA	11	0.686	0.46	0.00	NA	White British/Irish
Ind-4	3.61	1.17	NA	9	0.945	0.56	0.36	NA	White British/Irish
Neg-1	2.26	0.549	58	NA	0.532	0.41	1.06	0.124	White British/Irish
Ind-5	2.59	0.512	71	NA	3.441	0.41	2.30	0.148	White British/Irish
Median [IQR] values for all anti-HBc positives and negatives identified from in-house anti-HBc blocking assay									
Positives (n=308)	6.34 [3.88-7.37]	9.68 [4.64-14.50]	87 [81-93]	79 [69-85]	-	0.95 [0.62-3.03]	471 [95-2000]	0.710 [0.566-0.805]	-
Negatives (n=78)	1.45 [1.13-1.84]	0.502 [0.443-0.735]	9 [-23-24]	NA	-	0.43 [0.40-0.53]	1 [0-53]	NA	-

Table 17.[5] Serological and demographic characteristics of the eleven inconclusive samples identified from the in-house blocking assay. Samples are labelled according to their final status: Murex blocking assay +/- Monalisa assay (Pos), not blocked on the Murex and negative on Monalisa (Ind), Murex-negative and positivity on Monalisa only (Ind), and Murex-negative and Monalisa-positive (Neg). Underlined values indicate positive values according to the assay protocols. Average ratios are shown in the table. NA indicates where the blocking and avidity assays were not done since anti-HBc was negative on the assay. *Where anti-HBs titres were >1000 IU/L, 2000 IU/L was used as an approximation to obtain average values.

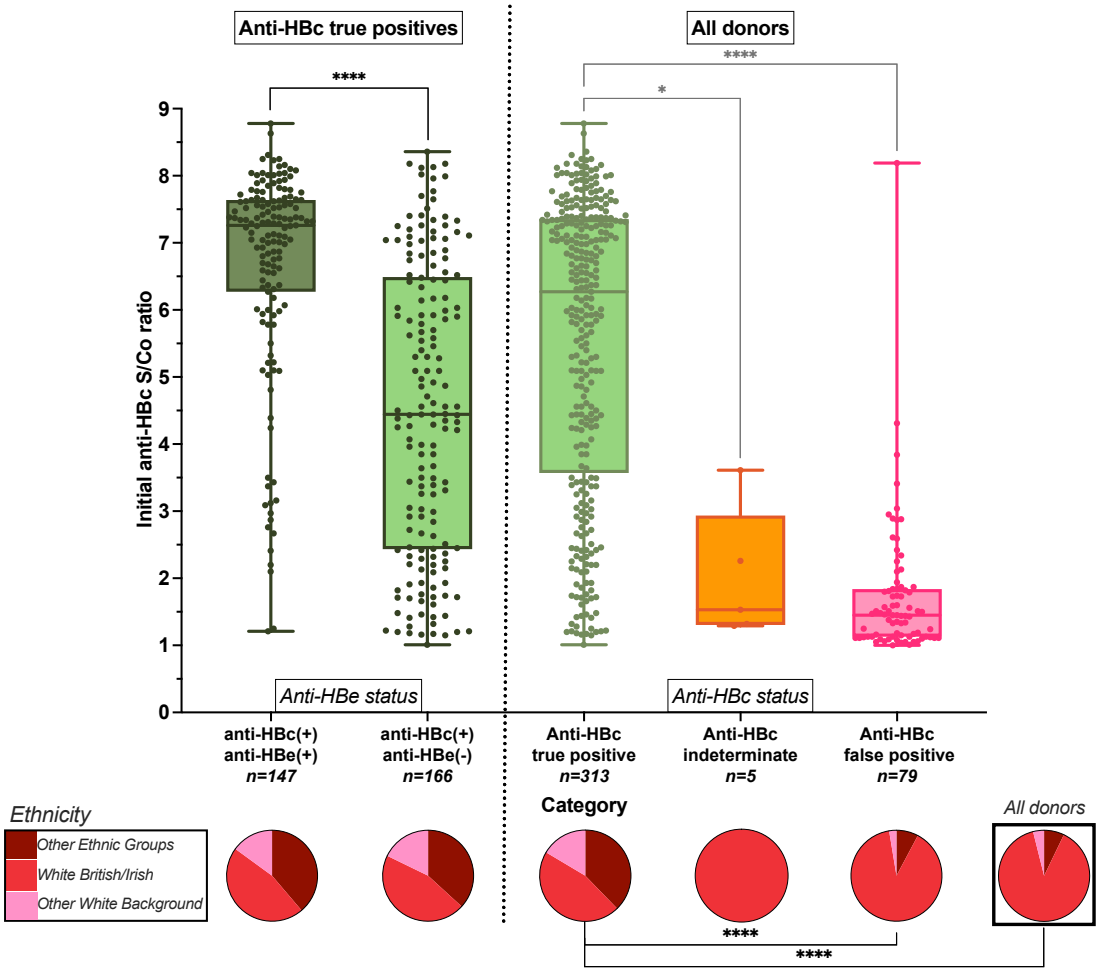
HBV DNA detection by individual NAT

Individual NAT identified one HBsAg-negative donor with anti-HBc antibodies positive for HBV DNA (Ct 36.4; IU/mL unavailable) with anti-HBs >100 IU/L from 397 tested. This donor was known to have recently received an HBV vaccine booster.

Characterisation of anti-HBc reactivities

Comparisons of initial anti-HBc screening ratios between anti-HBc confirmed positives, indeterminates, and false positives revealed significant differences between groups in anti-HBc reactivity ($p<0.0001$; Figure 30). There were significantly higher S/CO ratios for anti-HBc true positives (6.3 [4.0-7.4]) compared to anti-HBc indeterminates (1.5 [1.2-2.1]; $p<0.0001$) and anti-HBc false positives (1.5 [1.1-1.9]; $p<0.0001$) by post-hoc Dunn's test, whereas anti-HBc ratios between anti-HBc indeterminates and false-positives were similar ($p>0.999$). Comparisons of anti-HBe status in anti-HBc confirmed positive donors showed significantly higher anti-HBc ratios in anti-HBe positive than negative donors (7.3 [6.3-7.6] vs. 4.6 [2.6-6.5] respectively; $p<0.0001$).

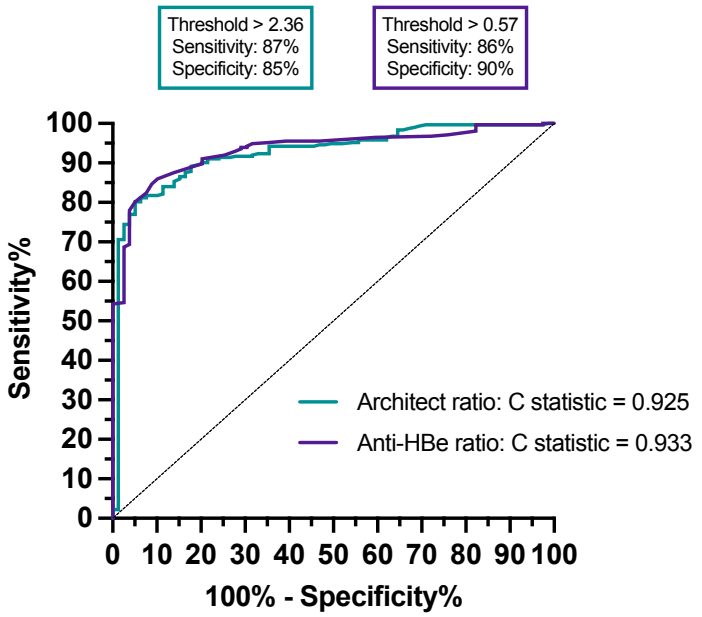
Figure 30.[5] Comparisons of median [interquartile range] initial anti-HBc signal to cut-off (S/CO) ratios between the 397 donor samples categorised by anti-HBc genuine positivity, false positivity, and indeterminate following the extensive testing detailed in Figure 2. The true-positive samples are further classified by anti-HBe status on the left hand of the graph, separated by a dotted line. The proportion of minority ethnic group donors for each category of donors is indicated, as well as from all donors in 2021. The Mann Whitney-U test compared anti-HBe status (darker coloured line), whilst the Kruskal-Willis test with post-Hoc Dunn's compared between positivity categories (light coloured lines), and Chi-squared tests compared ethnic groups, where *** denotes p<0.001 and **** denotes p<0.0001.



Predictors of anti-HBc true positivity

The value of anti-HBc reactivity in the Architect screening testing and the presence of anti-HBe to predict true and false anti-HBc positivity were determined quantitatively by ROC analysis using anti-HBc Architect and anti-HBe sample-to-control ratios. An Architect ratio of 2.4 showed 87% sensitivity and 85% specificity in predicting anti-HBc true positivity (Figure 31), whilst an anti-HBe cut-off of 0.6 would have 86% sensitivity and 90% specificity.

Figure 31.[5] Receiver operator characteristic (ROC) analysis to investigate Architect S/CO ratio (green) and anti-HBe ratio (purple) thresholds that may predict anti-HBc true positivity. For this analysis, anti-HBc true positives and true negatives (false positives) were utilised from this study's 397 samples, excluding the five indeterminate samples previously shown. The 90%/95% sensitivity and specificity threshold values for anti-HBc and anti-HBe are shown in the tables below the plot and 100% specificity thresholds for anti-HBc positivity.



Sensitivity (%)	Specificity (%)	Threshold anti-HBc S/Co ratio
95	53	1.48
90	80	2.10
87	85	2.36
82	90	2.88
77	95	3.40
2	100	8.21

Sensitivity (%)	Specificity (%)	Threshold anti-HBe S/Co ratio
95	68	0.47
90	80	0.55
86	90	0.57
80	95	0.60
54	100	0.83

Ethnic group comparisons

Sixty-one per cent of the 397 HBsAg-negative but anti-HBc repeat reactive donors were male, and 39% were female. Donors' median age was 47 [36-59]. Fifty-four per cent described themselves as White British/Irish ethnicity, 13% as other White ethnicities, five donors did not disclose their ethnicities, and the remaining 31% as other ethnic groups. The proportion of minority ethnic groups in anti-HBc true-positive donors was significantly higher than their representation among all blood donors (38% vs. 11%; $p<0.0001$; Figure 30). The proportions of 'Other Ethnic Groups' (38%) and 'Other White Background' (17%) donors in anti-HBc true-positive donors were also significantly higher than in anti-HBc indeterminate (11% minority ethnic groups and 0% Other White Background; $p<0.0001$), respectively; and anti-HBc false-positive donors (7% minority ethnic groups and 3% Other White Background; $p<0.0001$). Indeterminate and false-positive groups showed similar ethnic backgrounds ($p=0.686$), and both groups showed similar proportions of minority groups to those of all blood donors ($p=0.733$ and $p=0.842$, respectively).

Rare blood groups

Frequencies of selected blood groups were determined in the 981 anti-HBc positive donors with anti-HBs ≥100 IU/L identified in England's first six months of anti-HBc screening. Of these, 301 (31%) had a rare blood type. There was a significantly higher proportion of rare blood group donors of White Other and 'Other Ethnic Groups' in anti-HBc positive donors than in negative donors (29% vs. 7%, $p<0.0001$; and 31% vs. 26%, $p=0.033$; Table 18). Although there were similar proportions of R2R2 donors across ethnic groups ($p=0.819$), there were significantly more Ro donors in anti-HBc positive donors than negative donors (8% vs 3%, $p<0.0001$) where the proportion of 'Other Ethnic Groups' Ro donors was 19 times that of White British/Irish donors. There was also a significantly higher proportion of rr donors of White Other ethnicities in anti-HBc positive than all donors (25% vs 2%, $p<0.0001$).

	Ethnic group			
	White British/Irish (n, %)	**White Other (n, %)**	**Other Ethnic Groups (n, %)**	**All groups (n, %)**
All rare blood types				
Anti-HBc pos	119 (31%)	67 (29%)	115 (31%)	301 (31%)
Anti-HBc neg	200019 (29%)	2666 (7%)	18436 (26%)	221121 (28%)
p-value	*0.431*	*<0.0001*	*0.033*	*0.050*
R2R2				
Anti-HBc pos	8 (2%)	6 (3%)	6 (2%)	20 (2%)
Anti-HBc neg	13805 (2%)	779 (3%)	1159 (2%)	15743 (2%)
p-value	*0.855*	*0.46*	*>0.999*	*0.819*
Ro				
Anti-HBc pos	3 (1%)	3 (1%)	82 (19%)	88 (8%)
Anti-HBc neg	15372 (2%)	971 (2%)	10494 (15%)	26837 (3%)
p-value	*0.055*	*0.385*	*0.014*	*<0.0001*
rr				
Anti-HBc pos	108 (28%)	58 (25%)	27 (7%)	193 (20%)
Anti-HBc neg	170842 (25%)	916 (2%)	6783 (10%)	178541 (22%)
p-value	*0.14*	*<0.0001*	*0.156*	*0.039*

Table 18.[5] Comparisons of the proportion of ethnic groups between anti-HBc positive donors (n=981) and anti-HBc negative donors (n=794,861) for all rare blood types and each Rh blood type: R2R2, Ro and rr. The number of donors in each ethnic group for each blood type is shown, as well as the proportion of those donors representing all donors in that ethnic group for anti-HBc positive or negative donors. Fisher's exact tests were used to compute p-values.

Discussion

This chapter investigated the specificity and effectiveness of current screening and confirmatory testing of donors for anti-HBc antibodies. The association between anti-HBc blocking and anti-HBc confirmation in the Murex assay demonstrated the latter's efficacy in delineating true and false anti-HBc positive donors. Compared to the 80% specificity of the Architect assay, confirmation in the Murex assay reduced false positivity to around 1% of initially reactive donors (4/317) and 1% false negativity (1/80). Confirmation of anti-HBc reactivity showed a positive predictive value of 99% on Architect anti-HBc positive samples. Determination of the true seroprevalence of anti-HBc positivity in donors enabled the investigation of the effects of anti-HBc positive donor exclusion on the blood supply.

Specificity of anti-HBc testing is important and will help in communicating to donors if they have had a past HBV infection and providing appropriate advice about their future risk of developing HBV-associated progressive liver disease. HBV infection still carries a stigma, and many individuals deny having had a previous HBV infection, especially when the source of infection might simply relate to them being born in HBV high-endemicity areas. Our application of blocking and avidity assays showed that positivity in two anti-HBc assays strongly predicted true anti-HBc positive status, irrespective of assay S/CO ratios or anti-HBe/anti-HBs status. These results are highly relevant in interpreting isolated anti-HBc reactivity in donors or patients in general. Supplementary assays such as anti-HBc blocking may differentiate between past HBV infection and false reactivity in HBV-naïve donors[178] and provide an IgG-specific measurement that may increase specificity compared to assays measuring total anti-HBc. Testing algorithms using two different anti-HBc assays

would not only reassure blood donors but would also help clinicians who need to balance their decisions on starting antiviral prophylaxis based on isolated, non-confirmed anti-HBc results only, as recommended by the European Association for the Study of the Liver[20]. This testing algorithm may also be validated in other settings with alternative anti-HBc assays to provide equivalent confirmation of anti-HBc reactivity. Another strategy to identify false reactive donors would be utilising a threshold value[170]. We found that below a cut-off value of 2.36, the Architect anti-HBc II assay showed high sensitivity and specificity in predicting anti-HBc true reactivity. Using a threshold value is supported by our finding that samples with higher anti-HBc titres showed consistently higher high functional affinity (avidity); the demonstration of maturity of anti-HBc antibodies is consistent with genuine past HBV infection.

The rate of anti-HBc false reactivity of 20% on the Architect anti-HBc II assay, found in this study following extensive testing, is much higher than the 6% rate in a previous study in a smaller population of 79 initially reactive samples[179]. Our rate is comparable to a German study of 22% using a confirmatory strategy of two older anti-HBc platforms[169], suggesting minimal changes in the false positivity rate of anti-HBc screening assays over time despite claimed improvements.

Our findings also show that all anti-HBe positivity was completely concordant with confirmed anti-HBc positivity and that anti-HBe positivity can, therefore, be utilised as a specific marker for past HBV infection[168] but not as a marker for OBI since most OBI cases are anti-HBe negative[2]. However, we suggest that the 0.6 to 1.0 range of anti-HBe results may constitute a zone of uncertainty: an anti-HBe S/CO threshold of 0.57 had high sensitivity and specificity in predicting anti-HBc true

reactivity, and all donations with high 'negative' anti-HBe ratios above 0.83 were genuine anti-HBc-positives. Further, negativity in the anti-HBe assay has less predictive value; based on anti-HBc confirmatory testing, we found that the sensitivity of anti-HBe as a biomarker to identify resolved infection was limited. This may result from an increased prevalence of HBeAg-deficient HBV variants[170], the natural disappearance of detectable anti-HBe antibodies following recovery from infection, and the possibility that anti-HBe antibodies may never develop during the natural history of HBV infection[180].

A further question is whether deferring anti-HBc-positive donors significantly impacts the supply of units with rare blood groups. We found that 38% of anti-HBc confirmed positive donors (in the absence or presence of other HBV markers) comprised minority ethnic groups, consistent with previous information from donors originating from countries with endemic patterns of HBV infection[181]. These donors are more likely to be Rh type D positive[182]; we additionally found that rare Rh phenotypes were more common in anti-HBc-positive donors from minority ethnic groups, whilst the proportion of rare groups in White British/Irish anti-HBc positive donors was comparable to HBV-unexposed donors. The extensive minority ethnic group background of deferred anti-HBc positive donors with high anti-HBs levels (≥100 IU/L) is more likely to have the rare Rh phenotypes Ro and rr that are critically needed for patients with sickle cell disorder to lower the risk of alloimmunisation.

The potential acceptance of anti-HBc-positive blood donors with high anti-HBs titres has been previously considered a safe means to maintain an adequate blood supply with sufficient rare blood types. However, we found one anti-HBc positive

donor with high anti-HBs levels (vaccine-boosted) of 'Other Ethnic Groups' to have detectable viraemia out of 397 donors screened for HBV DNA, although whether infectivity in this donation would have been neutralised by high levels of anti-HBs remains unknown. It is important to note that HBV transmission from anti-HBc-positive donors with high anti-HBs levels is rare and hence assumed to be a very low risk[40].

In conclusion, the development and application of anti-HBc blocking and avidity assays combined with testing for anti-HBe demonstrated high positive (99%) and negative (99%) predictive value of the Murex anti-HBc assay for confirming donors reactive from screening with the Abbott Architect assay. Indeed, a relatively simple confirmatory algorithm with two anti-HBc assays would almost eliminate false-positive results. The impact of anti-HBc screening and excluding all positives to rare blood group types needs further consideration. Gaining insight into the screening and deferral strategies for OBI in blood establishments worldwide may help guide best practices.

Chapter 4: International review of blood donation screening for anti-HBc and occult hepatitis B virus infection

Most of this chapter is based on the published paper:

Fu MX, Faddy HM, Candotti D, et al. International review of blood donation screening for anti-HBc and occult hepatitis B virus infection. Transfusion. 2024 Nov;64(11):2144-2156. doi: 10.1111/trf.18018.

Introduction

Most anti-HBc assays have a relatively high false-reactivity rate, as shown in the previous chapter, where additional anti-HBc testing of initial reactive results may be necessary to limit unnecessary donor loss. Anti-HBc positivity alone does not equate to infectivity and will lead to the deferral of donors who do not pose a risk for recipients at all[1]. The impact of the deferral of such donors on the blood supply sufficiency means that anti-HBc screening is not feasible in countries where HBV is highly endemic[41], and it may also impact the supply of blood with rare red cell antigens in countries with low HBV endemicity, such as the UK as shown in the previous chapter. Further, there seems to be a lack of consensus between blood establishments on how anti-HBc-positive donors and those with OBI are followed up. The risks of transfusion transmission from donors with OBI are becoming more apparent, and the uptake of anti-HBc screening is increasing. However, to our knowledge, there has not been a review of anti-HBc screening practices across blood establishments globally. This chapter provides a timely review of international screening practices for anti-HBc and OBI and the different practices for donor deferral.

Materials and methods

Survey development

The survey was developed and piloted with multiple co-investigators, and questions were further refined based on their feedback. Meetings with these co-investigators were held to ensure that questions were similarly understood. The survey had multiple sections, each designed to elicit specific information about anti-HBc screening and the occurrence of OBI. Donors with OBI are defined as those who tested negative for HBsAg and positive for HBV DNA in the blood, regardless of anti-HBc positivity. The survey consisted of initial demographic questions, followed by multiple-choice questions on the timing of anti-HBc and HBV DNA screening, assays used for HBV NAT, and confirmation or additional testing for anti-HBc. Following ascertainment of confirmatory algorithms for anti-HBc, data on anti-HBc-positive and OBI donors identified from screening in 2022 were then ascertained, as well as any reported transfusion-transmissions of HBV from donors with OBI between 2018 and 2022. The last section of the survey asked about deferral and follow-up strategies of anti-HBc-positive and OBI donors. Branching free-text and further multiple-choice questions followed certain multiple-choice answers throughout the survey. The complete survey in its Word version is found in Appendix 2. This investigation did not require ethical approval since it reviewed screening practices and summary data without donation or donor identifiers.

Survey distribution

Respondents were recruited via the International Society of Blood Transfusion (ISBT) working party on transfusion-transmitted infectious diseases, inviting voluntary participation at a time selected to avoid survey fatigue. It was explicitly stated in the invitation email that responses were welcome from all blood establishments, regardless of whether they implemented anti-HBc screening. Between September and December 2023, an initial email and two reminders were sent to members of the ISBT working party. Respondents self-completed the survey in their own time via Jisc Online Surveys or a Word version. Individual respondents were contacted by email if responses required clarification, and respondents were also asked to validate their responses before the data interpretation stage.

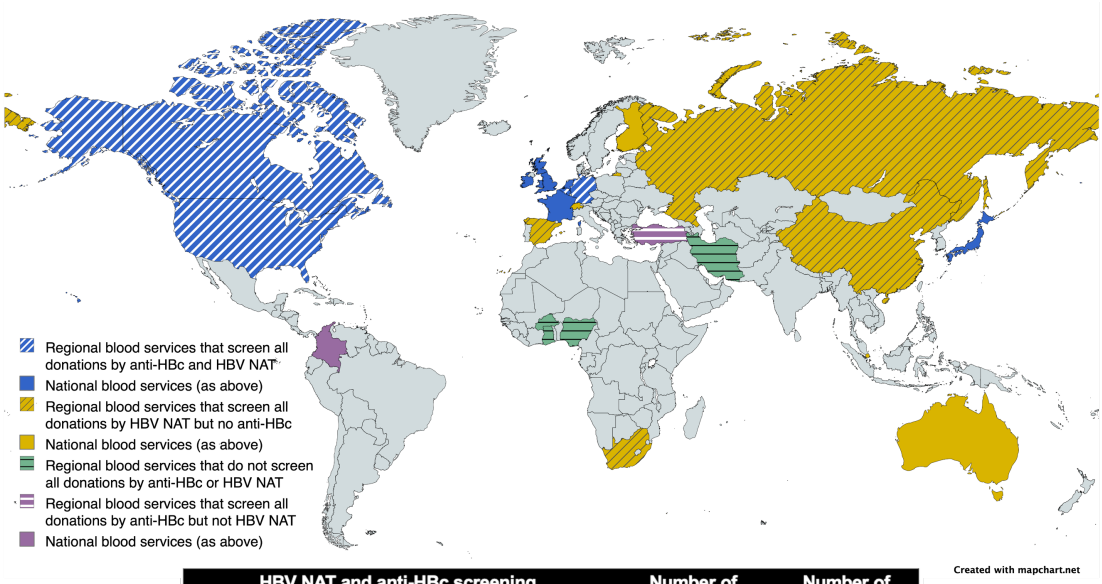
Data analysis

The population-level prevalence of HBsAg in each country was obtained[183], and OBI prevalence was aggregated from each participating country in this study. Because of non-normality in the distribution of HBsAg prevalence (tested using the Shapiro-Wilks test), this data was displayed as median [range]. Descriptive analyses were performed in Microsoft Excel. Fisher's exact tests and linear regression were performed in GraphPad Prism (version 10.2.0, LLC). Mapchart.net was used to create a world map showing the locations of participating countries. 'Respondent' refers to each response to the survey that represented a discrete data group, where certain respondents may represent more than one 'blood establishment' or blood collection organization, and each 'country' may have more than one respondent. Delineation of these terms allowed for the inter-respondent differences in testing and deferral strategies and respondents that collate data from multiple blood establishments to prevent data skew.

Results

Demographics

31 voluntary responses were received from 25 countries: Australia, Belgium, Burkina Faso, Canada, China, Colombia, England, Finland, France, Germany, Ghana, Iran, Ireland, Japan, The Netherlands, Nigeria, Russia, Scotland, Singapore, South Africa, Spain, Switzerland, Turkey, USA, and Wales. The response rate was roughly 60% from the invited working party. The working party represented 58 blood establishments from 39 countries. 44% of the countries involved in the working party were from LMICs, compared to approximately 70-80% of the world's countries being classified as LMICs. Responses were received from 6 of 17 LMICs and 19 of 22 HMICs in the working party. One respondent's data was a subset of another respondent's data from 84 blood establishments in Colombia; the first respondent's data was removed to avoid duplication, leaving 30 unique responses from 25 countries (Figure 32).



HBV NAT and anti-HBc screening	Number of respondents (%)	Number of countries (%)
All donations screened by anti-HBc and HBV NAT	14 (47%)	11 (44%)
All donations screened by anti-HBc but no HBV NAT	2 (7%)	2 (8%)
All donations screened by HBV NAT but no anti-HBc	10 (33%)	8 (32%)
No anti-HBc or HBV NAT screening	4 (13%)	4 (16%)

Figure 32.[6] Geographical locations of survey respondents, showing whether countries have regional or national respondents and delineating HBV screening strategies using differing colours. A summary table of anti-HBc and HBV NAT use for blood donation screening by participating respondents (n=30) and countries (n=25) is shown below the map. Blue indicates countries using anti-HBc and HBV NAT testing, purple indicates countries utilising anti-HBc testing without HBV NAT testing, and yellow indicates countries utilising HBV NAT testing without anti-HBc testing. Green indicates countries that do not perform either anti-HBc or HBV NAT testing of blood donations. This map was made with mapchart.net.

Data from this study based on screening in 2022 represents over 12,000,000 blood donors drawn from a population size of over 745,000,000 people. There were 12 (40%) respondents representing national and 18 (60%) representing regional blood establishments. Most respondents were from Europe (n=13; 43%), followed by Asia (n=7; 23%), Africa (n=4; 13%), North America (n=4; 13%), South America (n=1; 3%), and Oceania (n=1; 3%). All respondents (100%) tested for HBsAg, 24 (80%) utilised HBV NAT screening on all donations, and 16 (53%) utilised anti-HBc screening on all donations. Anti-HBc and HBV NAT were utilised by 14 (47%) to screen all blood donations, whilst 2 (7%) screened all donations for anti-HBc but not HBV NAT. 10 (33%) respondents screened all donations for HBV NAT but did not include anti-HBc in their initial screening, though targeted anti-HBc screening may have been performed. There were 4 (13%) respondents not screening for anti-HBc or NAT, citing economic constraints or lack of resources: three were in Africa and one in Asia. Of the total 12,113,490 donors, 10,000,190 (83%) were from establishments undertaking universal anti-HBc screening, 1,864,703 (15%) from establishments undertaking universal HBV DNA screening but not anti-HBc, and 248,597 (2%) from establishments with neither universal HBV DNA nor anti-HBc screening. 11 of 30 respondents (37%) performed pathogen inactivation on specific blood components; only one of seven LMIC countries performed pathogen inactivation whilst seven of eighteen (39%) high-income countries performed pathogen inactivation.

Anti-HBc and NAT screening

Sixteen respondents from thirteen countries performed universal anti-HBc screening. Collectively, these blood establishments screened over 10,000,000 blood donors in 2022 (range: 9969 to 2,578,552; Table 19). The total anti-HBc prevalence of participating blood establishments was 0.42% (35,980/8,539,019; a lower denominator since UK establishments began anti-HBc screening in the middle of the year), ranging from 0.01% in Ireland to 5.18% in Turkey (Table 20). Anti-HBc prevalence was six times higher in new donors (1.2% [13,711/1,154,972]) than in repeat donors (0.2% [12,537/6,416,624]; $p<0.0001$). HBV NAT was additionally screened in all donations for fourteen respondents from twelve countries (7,571,596 donors); pool sizes used for initial HBV NAT ranged from ID-NAT to 96 (limit of detection [95% LOD] ranging from 1.7 IU/mL to pooled 154 IU/mL; Table 20). Of the remaining two respondents who did not screen all donations for HBV NAT, one subsequently screened anti-HBc-positive donations with ID-NAT, and the other covered 84 blood establishments, of which 9 screened for HBV NAT.

Anti-HBc screening identified 182 anti-HBc-positive OBI donors, negative for HBsAg but with DNA detected by NAT screening (182/26,599; 0.7%), excluding the Colombia establishments where the number of OBI donors was not available. Through concurrent NAT screening, three donors from two blood establishments were identified as having anti-HBc-negative chronic OBI with sustained or intermittent detection of low HBV DNA levels on follow-up testing, where acute HBV infection was excluded. The collective OBI rate from all donors, combining HBsAg-negative and HBV DNA positive donors regardless of anti-HBc positivity, was 0.0024% in

respondents who performed universal anti-HBc screening with additional HBV DNA screening (185/7,571,596).

Table 19.[6] Demographic data for blood establishments that perform universal anti-HBc screening (n=16). For the country column, shaded cells represent respondents with regional data, whilst unshaded cells represent countries with national-level data. For the 95% LOD column, italicised values represent values obtained from the manufacturer's report, and non-italicized values represent values respondents reported but did not refer to the manufacturer. NA represents 'not applicable'. HBV NAT = hepatitis B virus nucleic acid testing; LOD = limit of detection; OBI = occult hepatitis B virus infection; FFP = fresh frozen plasma.

Country	Population of establishment area	Number of donors in 2022	New donors as % of all donors	Pool size used for HBV NAT before anti-HBc screening	95% LOD per donation (IU/mL)	Additional NAT after anti-HBc screening	Pathogen inactivation	OBI from all donors %
France	~66,000,000	1,434,393	17.5	ID	1.69	NA	Platelets and some FFP	0.0015
Japan	~120,000,000	2,578,552	12.6	ID	3.1	NA	No	0.0014
Ireland	5,280,000	74,163	9.1	ID	4	NA	No	0
Belgium**	~6,800,000	132,027	14.7	6	8.4	ID	Platelets and FFP	0
Canada 1	~30,000,000	375,059	19.6	6	*8.4*	No	Pooled platelets (only in one region)	0.0021
Canada 2	~8,600,000	128,334	19.6	16	80	No	No	0
Netherlands	~17,700,000	284,261	21.4	6	9	No	No	0.0014
Colombia[#]	24,438,137	967,423[#]	NA	#	NA	No	No	NA
England*	57,106,400	796,223	14.2	24	33.6	ID	No	0.0008
Scotland*	~5,400,000	94,355	9.9	24	33.6	ID	No	0.0088
Wales*	3,132,000	76,166	13.8	24	33.6	ID	No	0.0052
Germany 1	~13,400,000	257,569	19.2	96	154	ID	No	0
Germany 2	~3,000,000	18,844	21.0	48	67.2	ID	No	0
USA 1	~133,300,000	2,227,882	27.5	16	68.8	ID	Apheresis platelets	0.0038
USA 2	~100,000,000	544,970	25.3	16	73.6	ID	Apheresis platelets	0.0037

Turkey	~6,000,000	9969	9.0	Not done	NA	ID	No	0
Total	~600,000,000	10,000,190	13.7					0.0024

#Combined data from 84 blood establishments, 9 of which conducted HBV NAT. The number of donors was unknown, so the number of donations is shown in this table. Colombia data was not included in any collective analyses where data was unavailable.

*This table displays data for the whole of 2022. UK blood establishments commenced anti-HBc screening on 30 May (England), 6 April (Scotland), and 27 May (Wales). Data analyses were based on the number of donors screened for anti-HBc at each establishment from these dates until 31 December 2022. England and Scotland performed anti-HBc screening on all new donors, all returning donors, regular donors without a negative anti-HBc result, and donors with a newly identified HBV risk factor.

**Anti-HBc screening was performed on all new donors and was performed every two years since the first donation for repeat donors.

^Ireland takes a sample from new donors not born in the UK or the Republic of Ireland prior to the first donation

Table 20.[6] Number and proportion of anti-HBc positive donors, including those with OBI, for the survey respondents that perform universal anti-HBc screening, showing confirmation and deferral strategies applied in 2022 (n=16). Confirmation of anti-HBc positivity was performed on the donation sample unless specified as 'Repeat next donation', in which case confirmation was performed on a second specimen. Shaded cells mean 'not applicable'.

Country	Total number of anti-HBc positive donors	All donors anti-HBc positive (%)	New donors -anti-HBc positive (%)	Number of anti-HBc positive donors with OBI	Confirmation of anti-HBc positivity	Deferral of donors with unconfirmed anti-HBc result	Deferral of donors with confirmed anti-HBc result
France	2330	0.16	0.88	19[a]	Repeat same; second assay	Temporary deferral	Permanent deferral
Japan	7511	0.29	0.57	35	Not confirmed	Permanent deferral *	
Ireland	7	0.01	0.06	0	Repeat same; two further assays	Permanent deferral	Permanent deferral
Belgium	306	0.23	1.03	0	Repeat same assay	None	Permanent deferral **
Canada 1	1495	0.40	1.75	8	Not confirmed	Permanent deferral	
Canada 2	369	0.29	0.99	0	Repeat next donation	Temporary deferral	Permanent deferral
Netherlands	1179	0.41	0.68	3[b]	Repeat same assay	Temporary deferral *	Permanent deferral *
Colombia	9381[+]	0.97	-	-	Repeat next donation[=]	Temporary deferral	Permanent deferral
England^	403	#	0.33	4	Repeat same; second assay	Temporary deferral	Permanent deferral
Scotland	204	#	0.71	7	Repeat same; two further assays	Temporary deferral	Permanent deferral
Wales	91	#	0.33	2	Repeat same; second assay	Temporary deferral	Permanent deferral
Germany 1	355	0.14	0.60	0	Repeat same; two further assays	None	Permanent deferral
Germany 2	21	0.11	0.53	0	Repeat same; second assay	Temporary deferral	Permanent deferral
USA 1[-]	9596	0.43	0.90	85	Repeat same assay	None	Temporary deferral
USA 2[-]	2216	0.41	0.94	19	Repeat same assay	Temporary deferral	Permanent deferral
Turkey	516	5.18	2.01	0	Repeat same assay	Permanent deferral ***	Permanent deferral ***
Total	35,980	0.42	1.17	182			

^Anti-HBc was only confirmed for donors with anti-HBs < 100 IU/L in 2022 at this blood establishment, so results from anti-HBc positive donors with anti-HBs < 100 IU/L are shown.

#The UK blood establishments began anti-HBc screening in 2022, so a disproportionately larger proportion of repeat donors were screened anti-HBc positive. These figures are excluded from data analyses.

+The number of donors was unknown, so the number of donations is displayed here.

-Both of these participating respondents in the USA use the same centralised testing laboratory

=It may be possible that donors who tested anti-HBc positive in the index donation went to a different blood establishment using a different anti-HBc assay for their next donation; this was considered a true confirmation of positivity. If donors returned to the same or different blood establishment using the same assay, this was considered double reactivity and not true confirmation. Since data was unavailable for the true confirmed positive donations, the total number of anti-HBc positives is provided.

[a]Two extra donors were identified as seronegative OBI through NAT screening

[b]One extra donor was identified as anti-HBc negative OBI through NAT screening

* Allowed if anti-HBs >200 IU/L; otherwise, deferral

**Permanent deferral if anti-HBs > 0 IU/L:; temporary deferral if anti-HBs = 0 IU/L with control testing after one month and option for re-entry after the maximal window period of 6 months if all HBV markers are negative and anti-HBs titre remains at 0 IU/L.

***Allowed if anti-HBs >100 IU/L; otherwise, permanent deferral

NAT screening without anti-HBc

Ten respondents from eight countries performed HBV NAT screening without anti-HBc screening of all blood donations, where 95% NAT LOD ranged from 1.2 IU/mL to a pooled 8.4 IU/mL (Table 21). The collective OBI detection rate in these respondents was five times higher than in respondents performing universal anti-HBc screening (0.0125% versus 0.0024%; $p<0.0001$). The higher rate reflects both the lack of anti-HBc screening to defer donors with potential OBI before their intermittent viral loads became detectable by HBV NAT screening, as well as the greater propensity to avoid anti-HBc screening in HBV endemic countries, with a median HBsAg frequency of 2.4% [range 0.6-12.2%] compared to 0.9% [range 0.3-3.0%] in countries that have adopted anti-HBc screening.

Table 21.[6] Demographic data for blood establishments that perform HBV NAT screening of all blood donations but do not perform anti-HBc screening on all donations (n=10). Some blood establishments perform targeted anti-HBc screening: the blood establishments that provided this information are indicated, but this does not necessarily mean that the other blood establishments did not utilise a similar strategy. For the country column, shaded cells represent respondents with regional data, whilst unshaded cells represent countries with national-level data. For the 95% LOD column, italicised values represent values obtained from the manufacturer's report, and non-italicized values represent values respondents reported but did not refer to the manufacturer.

Country	Population of establishment area	Number of donors in 2022	New donors as % of all donors	Pool size used for HBV NAT	LOD per single donation (IU/mL)	Pathogen inactivation	OBI from HBV DNA	OBI from all donors %
Switzerland	8,815,400	255,024	10.6	ID	*1.2*	Platelets and 50% of FFP	13	0.0051
Finland	5,556,000	115,553	16.3	ID	1.4	No	2	0.0017
Singapore	4,149,253	91,139	25.1	ID	1.4	No	14	0.0154
China 1	6,087,000	58,687	46.9	ID or 6	3.4 / 13.8	FFP	38	0.0648
China 2	7,460,000	118,040	16.0	ID	3.3	Apheresis platelets and FFP	42	0.0356
Australia*	26,268,359	428,871	21.8	ID	4.3	No	19	0.0044
South Africa	~52,000,000	473,564	27.2	ID	*4.3*	No	89	0.0188
Spain 1	5,316,478	116,267	14.0	ID	4.3	Platelets and FFP	8	0.0069
Spain 2	6,859,914	177,558	22.7	6	*8.4*	20% platelets and FFP	9	0.0051
Russia	Unknown	30,000	13.3	ID or 6	*1.4 / 25.8*	Platelets and FFP	0	0.0000
Total	~122,500,000	1,864,703	26.9				234	0.0125

*Performed targeted anti-HBc screening for donors who reported a history of hepatitis.

Anti-HBc confirmation

The sixteen respondents have each implemented different confirmation strategies for anti-HBc reactivity: two (13%) did not confirm anti-HBc results on the index donation; five (31%) repeated testing using the same assay (although three of five respondents did not consider this as a true confirmation of reactivity); four (25%) used one further anti-HBc assay from a different manufacturer, and three (19%) used a third different anti-HBc assay to confirm initial positive results. As an alternative algorithm, two (13%) respondents re-tested the next donation for anti-HBc: one respondent re-tested using the same assay, and the other respondent retested in the same or alternative assay but did not consider retesting in the same assay as a true confirmation of reactivity (Table 20).

Deferral of anti-HBc positive donors

The two respondents who did not confirm anti-HBc results permanently deferred all anti-HBc-positive donors. Of the other fourteen that confirmed initial anti-HBc-positive results, nine temporarily deferred the unconfirmed positive donors and permanently deferred confirmed positive donors; two permanently deferred all positive donors regardless of confirmation testing, whilst the remaining three did not defer unconfirmed positive donors but either permanently or temporarily deferred confirmed positives. Four respondents also used anti-HBs titres to guide anti-HBc-positive donor deferral: three respondents allowed DNA-negative donors if anti-HBs titres exceeded either 100 IU/L or 200 IU/L. The fourth respondent temporarily deferred donors if anti-HBs titres were 0 IU/L (instead of permanent deferral if anti-HBs > 0 IU/L) with potential re-entry after the maximal window period if donors tested anti-HBc-negative and anti-HBs remained at 0 IU/L (Table 20).

Donor follow-up

For confirmed anti-HBc-positive donors, most respondents (81%; 13/16) provided results to the donor and their doctor, whilst three respondents did not follow up with anti-HBc-positive donors (Table 22). For donors with OBI, most respondents (80%; 20 of 25 respondents that screened for HBV DNA) communicated results to the donor and often to their doctor, and 32% of all respondents additionally referred the donors to a specialist. Three of the remaining five respondents did not provide OBI details to the donor but referred the donor to a specialist, whilst two did not organise any follow-up.

Apart from differences in donor deferral, six of 15 respondents performed the same follow-up for anti-HBc positive donors as OBI donors, while eight respondents performed more follow-up for OBI donors. One respondent provided positive anti-HBc results to their donors but did not perform any follow-up for donors identified with OBI.

Table 22.[6] Follow-up of anti-HBc positive and OBI donors amongst survey respondents.

Follow-up of blood donors with past or occult HBV infection	**Number of respondents**
Anti-HBc positive donors	
Results provided to donor and their GP/family doctor	13
No follow-up organised by the blood establishment	3
Donors with occult HBV infection	
Results provided to donor and their GP/family doctor	20
Referral to specialist	11*
Both referral to specialist AND results provided to donor and their GP/family doctor	*8*
No follow-up organised by the blood establishment	2

*One blood establishment referred donors only if their HBV DNA level is over 10 IU/mL.

Reported HBV transmissions from blood donors with OBI

Between 2018 and 2022, the study participants reported four HBV transfusion transmissions linked to three donors with OBI in low-endemic countries. A single HBV transfusion transmission was linked to an anti-HBc negative donor in the Japan blood establishment who tested ID-NAT-negative on screening, but a subsequent donation was identified as DNA-positive[94]. The increase in anti-HBs titres on follow-up suggests that the index donation may have been in the acute window period due to reinfection with HBV or vaccine breakthrough infection, but chronic anti-HBc-negative OBI with transient viraemia may also have been plausible. The other three transmissions reported were from the England blood establishment before the introduction of universal anti-HBc screening in 2022, where two anti-HBc positive donors with OBI with viral loads below the pooled NAT LOD transmitted HBV to three recipients in total[184]; no transmissions have been reported since the introduction of anti-HBc screening in England. Thus, with the universal anti-HBc screening strategy utilised by blood establishments between 2018 and 2022, only one transfusion transmission from an anti-HBc negative donor with unclear OBI status was reported. Of countries that did not perform universal anti-HBc screening, no HBV transmissions from donors with OBI were reported from 2018 to 2022.

Correlation of anti-HBc and OBI prevalence with HBsAg positivity

A weak positive correlation (r^2=0.1917) was observed between HBsAg prevalence and anti-HBc positivity (Figure 33A) for all donors, with no noticeable differences between new and repeat donors. A higher HBsAg prevalence correlated strongly with OBI prevalence (r^2=0.8064; Figure 33B).

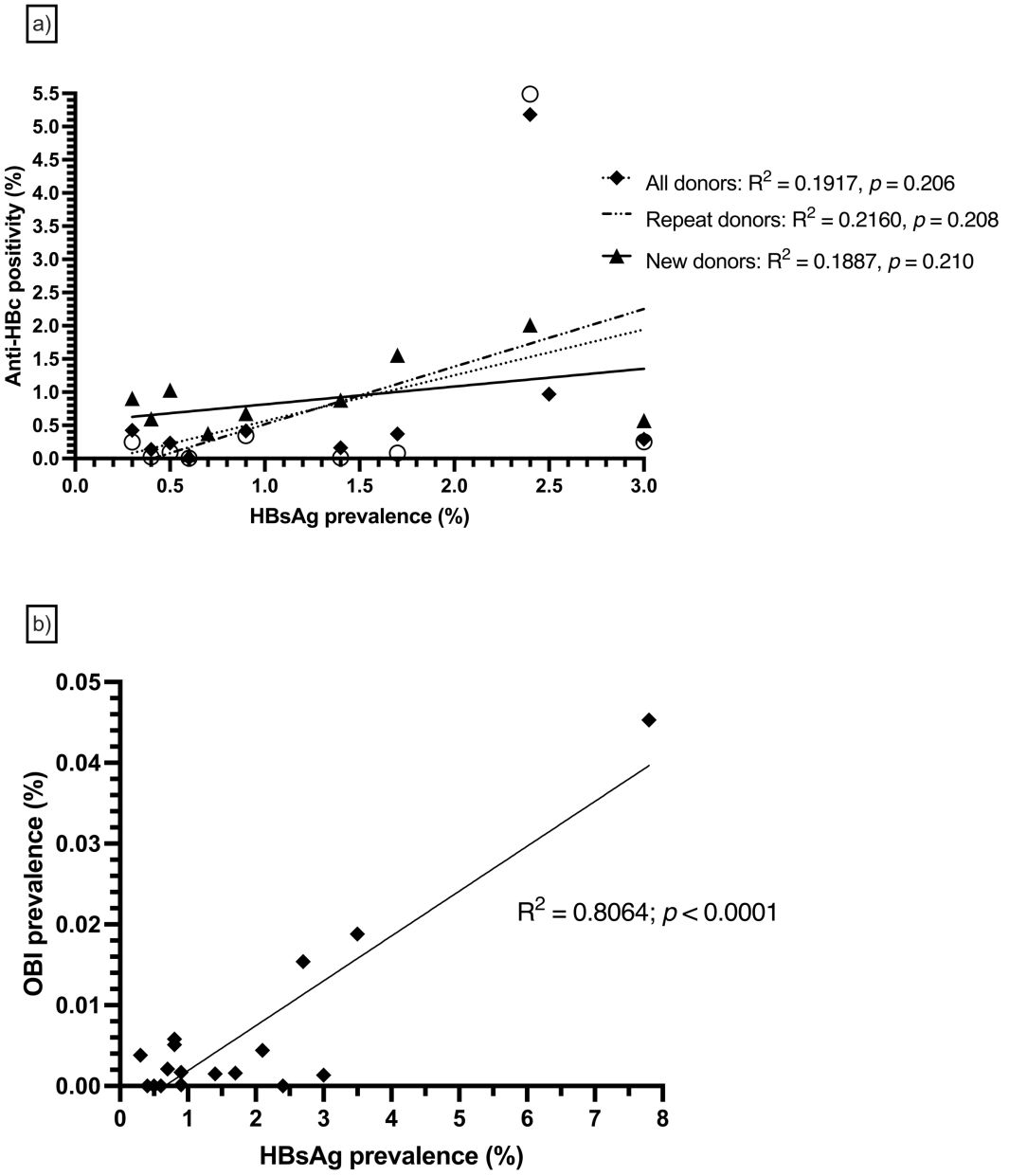


Figure 33.[6] Simple linear regression plots of country HBsAg positivity with a) anti-HBc positivity rate in all donors, new donors, and repeat donors for all countries that perform universal anti-HBc screening, and b) each country's reported OBI prevalence for all countries that screen using NAT.

Discussion

This international investigation draws data from various countries from all continents, covering over 12 million blood donors in 2022. Though many countries have implemented anti-HBc screening, there was considerable heterogeneity globally in screening and confirmatory algorithms and follow-up strategies for anti-HBc positive and OBI donors.

Our data suggests the effectiveness of anti-HBc screening and highly sensitive HBV NAT screening to prevent nearly all HBV transmission from donors with OBI. It is important to note that transmission of HBV from donors with OBI may be under-reported globally due to various reasons, including the asymptomatic nature of most HBV infections, lack of donor lookback investigations, and potential viral clearance at the time of lookback[1]. The significantly higher rate of anti-HBc positivity in new donors than in repeat donors suggests that selective screening strategies may be utilised if screening all donors for anti-HBc may be costly or result in a significant loss of donors due to false positive results. Some UK blood establishments screen for anti-HBc in all new and returning donors, regular donors without an existing negative anti-HBc result, and donors with a newly identified HBV risk factor[185]. However, anti-HBc screening would not detect infectious donors in the window period of acute HBV infection pre-seroconversion for anti-HBc and hence not classified as having OBI, documented to be 100-fold more infectious to transmit HBV[186,187]. Additional universal HBV ID-NAT screening with lower LODs may be needed to identify all potentially infectious donors with HBV. Alternatively, additional pathogen reduction methods, as already used by many blood establishments, could be valuable in increasing blood safety from low-level infectious agents[188] and may even be cost-

effective in low-income settings[189]. However, pathogen reduction is currently unavailable for all blood components and comes at the expense of the quality loss in blood components[190].

Sensitive NAT screening is also required to identify the small proportion of donors with chronic OBI who do not have detectable anti-HBc antibodies[1], corroborated by the anti-HBc negative chronic OBI donors found by two blood establishments in our study. The clinical significance of anti-HBc negative OBI and seronegative OBI for the donor remains largely unknown apart from a hypothesised higher infectivity. Through a case of an anti-HBc negative but anti-HBs positive donor with confirmed transfusion-transmission of HBV[94], this chapter highlights the need to be aware of the potential transmissibility of donors with this serological profile who may be either in the acute window period or in the occult phase of HBV infection, and who have been shown to constitute between 4.4%[76] and 11%[96] of donors with OBI in parts of Asia.

For low-endemic countries with low seroprevalence of anti-HBc where anti-HBc screening is feasible, the main screening problem arises from the high numbers of false positive results[167]. A recent study showed that confirmation of initially reactive results with one additional anti-HBc assay was necessary to identify true-positive anti-HBc results without compromising false-negativity[5]. Where 56% (9/16) of the respondents in this investigation did not utilise confirmation strategies to identify these anti-HBc positive donors, confirmation of initial reactive results using an alternative assay would reduce the donor loss; an estimated 20% of donors with initially reactive but unconfirmed anti-HBc results[5] may not need to be deferred permanently.

It has also been shown that many anti-HBc-positive donors in low-endemic settings are of rare blood donor groups[5], highlighting the loss to the sustainability of the blood supply through deferring anti-HBc-positive donors when this chapter shows that only 0.7% of all anti-HBc-positive donors in low-endemic countries have detectable HBV DNA and be potentially infectious. Some blood establishments accept anti-HBc-positive donations with high anti-HBs titres based on the idea that anti-HBs would neutralise any HBV infectivity, and this is supported by the current lack of documented transmissions from DNA-positive donations with anti-HBs titres greater than 100 IU/L. However, caution must be exercised when these blood components are transfused to immunocompromised recipients[1], and this approach may be more relevant for chronically transfused patients who need highly matched red blood cell transfusions. Nonetheless, the fluctuating HBV viral loads below the LOD of NAT assays found in OBI donors, and the potential infectivity of anti-HBc-positive only donors[38] suggests the deferral of anti-HBc-positive donors to ensure blood safety.

Despite the enhanced safety of anti-HBc screened blood, deferral of positive donors becomes unproductive in areas of high HBV endemicity, where large proportions of the population may have anti-HBc antibodies, exceeding 50% in some cases[82]. However, endemicity is also associated with higher frequencies of OBI, five-fold higher in donors screened in the higher-endemic countries in the current chapter. Reduction of HBV transfusion-transmission risk, therefore, requires HBV NAT screening and avoiding deferral of anti-HBc-positive donors, where modelled and empiric data has shown a decline in the residual risk of transfusion-transmission of OBI blood components post-NAT implementation[191]. While blood establishments in

Asia and South Africa screen for HBV DNA using relatively sensitive NAT, most African blood establishments only screen for HBsAg despite having the highest rate of chronic HBV infection worldwide[183]. Although many individuals may have anti-HBs antibodies to provide some protection in high-endemic countries, the strong correlation between OBI prevalence and HBsAg positivity implies a substantial and ongoing likelihood of high rates of HBV transfusion transmission from HBsAg-negative OBI donors. Systematic screening for HBV DNA may be necessary in these countries in line with the World Health Organization's (WHO) viral hepatitis elimination targets[192]. Although population-level immunity from prior exposure and vaccination may reduce susceptibility to infection in some recipients, protection is heterogeneous and incomplete, particularly in immunocompromised patients. Recipient-based strategies, such as vaccination of frequent transfusion recipients and antiviral prophylaxis of transplant recipients, may reduce individual risk but are not immediately protective and may not mitigate transmission from low-level viraemia at a population level. Consequently, HBV NAT remains the most effective intervention for reducing transfusion-transmission risk in high-endemicity settings.

In line with the WHO's goals of eradicating HBV, blood establishments may also have a role to play in contributing to the public health response by helping with donor counselling. Our results showed considerable heterogeneity in the deferral strategies of donors who tested anti-HBc-positive and those with OBI, where providing results and counselling to donors for blood establishments that do not yet do so may be helpful towards viral elimination targets. Although most blood establishments provide positive anti-HBc and OBI results to the donor, there is little professional consensus among hepatologists on the necessity of referring donors with

OBI to a specialist; definitive evidence on the long-term adverse effects of OBI is scarce and warrants further investigation. It is also noted that referral pathways vary globally[193] and may be influenced by differing healthcare models.

Response rate was high but were predominantly from more developed countries, reflected by the lower response rate from LMICs and the greater representation of high-income countries in the working party. However, the proportion of respondents not performing HBV NAT (13%) was similar to a previous NAT survey from the working party (16%)[194]. Although responses were received from every continent, the bias towards more developed countries may have affected the generalisability of our findings to blood establishments globally, but it is hypothesised that many non-responders do not perform screening for OBI. This may also underestimate the aggregated anti-HBc prevalence. However, the aggregated figure includes both initial reactive and confirmed reactive results due to the heterogeneity in confirmatory anti-HBc testing, and so may overestimate the true reactive aggregated anti-HBc rate in the responses.

Further, the reported detection rate of OBI is highly likely to underestimate the true number of donors with OBI for various reasons. Viraemia is often only intermittently detectable in donors with OBI[17], and HBV DNA was often only detected by single NAT assays at single time points in our study. Moreover, viral loads in OBI are commonly at, or below, the sensitivities of currently available NAT assays[191], where some respondents did not perform ID-NAT, and sensitivities of ID-NAT assays varied. More cases of true persistent OBI infections would be detected if replicate ID-NAT testing[17], high-volume plasma extraction with ultrasensitive NAT[2], or testing of

sequential donations were performed[17]. Conversely, although OBI was explicitly mentioned in the survey questions, it may have been possible that respondents included donors with HBV infection in the acute window period for the numbers of OBI donors since OBI was defined as HBsAg-negative and DNA-positive in the survey.

In conclusion, this chapter highlights the importance of anti-HBc or sensitive ID-NAT screening globally to reduce the risk of transfusion-transmitted HBV infection from donors with OBI, with the acknowledgement that OBI donors who test anti-HBc negative or even ID-NAT-negative may still pose a risk of transmission. The lack of confirmation of anti-HBc results may lead to the unnecessary deferral of donors in many blood establishments, where further evidence is needed to affirm the optimal referral pathway of anti-HBc positive and OBI donors. Notably, introducing HBV DNA screening in high-endemic areas is essential for identifying OBI donors and reducing transfusion transmission of HBV infection.

Synthesis chapter

Parts of this chapter are based on the published papers:

Fu MX, Simmonds P, Harvala H. A need for confirmatory testing of isolated HBcAb-positive results in screening programs. Hepatol Commun. 2024 Oct 3;8(10):e0554. doi: 10.1097/HC9.0000000000000554.

Fu MX, Elsharkawy A, Healy B, et al. Occult hepatitis B virus infection: risk for a blood supply, but how about individuals' health? EClinicalMedicine. 2025 Feb 1;81:103095. doi: 10.1016/j.eclinm.2025.103095.

Fu MX, Simmonds P, Andersson M, Harvala H. Biomarkers of transfusion transmitted occult hepatitis B virus infection: Where are we and what next? Rev Med Virol. 2024 Mar;34(2):e2525. doi: 10.1002/rmv.2525.

Major findings and implications of the thesis

OBI is known to be a challenging clinical entity to diagnose, due to the absence of HBsAg detectability, and the presence of extremely low and fluctuating VLs. The need for accurate diagnosis in blood transfusion is imperative, considering the risk of transfusion of blood components infected with OBI to immunocompromised recipients. Testing of HBV DNA using ultrasensitive assays would enable an accurate identification of infectious donations. The first chapter showed the development of an ultrasensitive PCR assay to detect HBV DNA through increased extraction volume, sensitive PCR primers and conditions, as well as increased DNA input. Utilisation of this method enabled the identification of four times the number of DNA-positive blood donations when compared to the standard DNA testing system used by NHSBT. This sensitivity would be improved even further by PEG extraction. Whilst this method could be utilised in blood donor screening, the manual and time-consuming

methodology hinders its immediate feasibility for use in the real world. Selective sensitive HBV DNA screening of blood donations may be possible, where the first chapter also showed that higher anti-HBc titres could be predictive of HBV DNA presence. These results could be applied further. Firstly, investigations into increasing input volume in commercial PCR assays could be beneficial for blood donor screening, as this would mean a similar processing time but potentially increasing sensitivity of detection. Secondly, investigations into additional biomarkers that could more sensitively identify donations with OBI would be beneficial, as these could be combined with anti-HBc titres in creating a multivariate diagnostic algorithm to better identify donors with OBI. Potential biomarkers are further discussed in the final section of this thesis.

NGS technologies have been revolutionising the field of microbiology and transfusion science. However, its use for screening blood donations for transfusion-transmitted infections carries debate. Whilst the ability to screen multiple pathogens in a single test seems advantageous, the sensitivity of NGS methods may be less than real-time PCR methods, and NGS protocols can be cumbersome and require experienced personnel. In the second chapter of the thesis, where multiple NGS protocols were compared, it was shown that the sensitivity of NGS to detect HBV DNA was insufficient to detect those at the lowest VLs, which could be detected by real-time PCR. Even with a PCR pre-amplification step prior to NGS library preparation, there was either a lack of sensitivity and/or evidence of background contamination, which prevented the detection of HBV DNA in the lowest VL samples. Whilst the ability to screen for multiple pathogens in one test was evidently useful, the presence of many of these pathogens in otherwise healthy individuals may not be of

significance or reliability, and could unnecessarily confuse and worry donors and healthcare providers. Therefore, screening using NGS in the blood donation setting may be useful when the aim is to screen for multiple pathogens; time- and cost-effectiveness comparisons with standard real-time PCR screening will be necessary before their potential introduction. In other clinical contexts, such as investigations into unknown pathogenic causes of symptomatic infection, the sensitivity of NGS technologies would likely detect most, if not all, causes, based on the HBV DNA results observed here. In the context of HBV (and potentially other viruses such as HIV), where VLs can be extremely low, real-time PCR is still needed as a more sensitive screening tool. However, commercially available PCR assays are still of insufficient sensitivity to detect the VLs at extremely low values. Combined with the comparably high cost of HBV DNA screening as modelled by the UK's SaBTO, there is a clear rationale for anti-HBc screening to identify and exclude HBsAg-negative blood donations that may contain extremely low levels of HBV DNA (or from donors infected with OBI).

However, anti-HBc screening comes with issues such as false-positive results and the fact that many anti-HBc positive donations do not contain HBV DNA, meaning a large number of donors may be deferred but they may not actually present a risk to blood safety. In the third chapter of the thesis, through the development of an in-house anti-HBc assay along with testing with multiple other commercial assays, it was shown that a two-assay anti-HBc strategy (namely the Architect assay followed by the Murex assay) had a 99% true-positive and true-negative detection rate, whilst solely using the first assay had only a 79% true-positive rate when screening consecutive blood donations from NHSBT. Despite this, less than half of the surveyed blood

establishments in the fourth chapter performed adequate confirmatory testing using a second assay. The low positive predictive value (PPV) of current anti-HBc assays should be noted in international guidelines. When large populations with low anti-HBc seroprevalence are screened, high false positivity rates could outnumber true diagnoses. Mass screening would result in increasing numbers of individuals wrongly identified as positive. This issue becomes more urgent as community-based seroprevalence testing may be pursued more globally to achieve HBV elimination goals, especially when the United States Centers for Disease Control and Prevention (CDC) has recently recommended a one-time universal screening of all adults for HBV infection with three laboratory tests: HBsAg, anti-HBs, and anti-HBc[195]. Screening for anti-HBc antibodies without measures to exclude false-positive results could affect a large number of otherwise healthy individuals with no history of HBV infection. Individuals who lack an understanding of resolved HBV infection may experience anxiety and distress due to the negative public perceptions surrounding HBV positivity. It seems necessary for screening programs to introduce measures to identify and reduce notification of false-positive anti-HBc results. In support of a gold-standard testing strategy for anti-HBc, an implication of this chapter's finding is that reflex testing should be performed on initially isolated anti-HBc positive specimens (with or without anti-HBs from vaccination) using a different supplemental anti-HBc assay of similar sensitivity. Negative results in either assay would indicate anti-HBc negativity, whilst positivity in both assays would confirm true anti-HBc positivity. This strategy would be similar to the two-assay testing strategy recommended by the WHO for HBsAg screening in low-prevalence settings[196]. With any gold-standard testing algorithm, there is a need for appropriate communication of false-positive results to minimise the psychosocial impact of notification, as detailed in this referenced editorial[197]. However,

anti-HBc testing excludes a large number of otherwise healthy donors who do not have circulating HBV DNA; this results in a large number of donors with rare blood groups who are excluded from donation, as also found in the third chapter. This negatively impacts the sustainability of the blood supply, and a more sensitive screening strategy may be needed to identify donors at a greater risk of having OBI and result in fewer donors being deferred. Beyond donor-centred screening, recipient-focused risk-reduction strategies may warrant consideration. These could include targeted antiviral prophylaxis or HBV vaccination in recipients anticipated to require transfusion(s).

Whilst anti-HBc screening is effective in low-endemic countries where HBV exposure is relatively low, anti-HBc screening becomes unfeasible in high-endemic countries. A global systematic review is underway to investigate the prevalence of anti-HBc in different WHO regions and specific countries, as well as stratifying by population group and investigating the changes in anti-HBc prevalence over time. With input from patient and public involvement (PPI) contributors with diverse experiences of HBV infection and ethnicity, the results of this study would be imperative for the WHO elimination goals and would be informative for blood establishments considering the introduction of anti-HBc screening. Currently, individual HBV DNA testing would be necessary for higher-endemic countries, as shown by the fourth chapter. This is not optimal, as shown by the first chapter, where VLs in OBI-infected donors are often below the sensitivity of NAT assays. This reinforces the case to investigate alternative markers that may assist in stratifying donors more at-risk of OBI. Alternatively, pathogen inactivation (not yet available for all blood products) would likely reduce the risk of OBI, which is already implemented in certain higher-endemic countries such as China, as found in the international survey.

The low prevalence of HBV DNA in HBsAg-negative, anti-HBc positive blood donors in low-endemic countries was highlighted by the findings in the fourth chapter of the thesis (0.68%), but with a significant 0.42% of all blood donors testing anti-HBc positive and deferred from donation. Despite individual NAT and anti-HBc testing, the Japanese blood establishment reported a transfusion-transmission from an anti-HBc negative donor, highlighting the complexity of detecting OBI. Further, this chapter showed the heterogeneity in the follow-up of donors identified as positive for anti-HBc or OBI, where the optimal referral pathway requires ascertainment. To this effect, multidisciplinary discussions were held between UK blood establishments, hepatologists, and public health experts to discuss the care and management for blood donors with OBI. There was no professional consensus on the need to follow up with OBI donors. From the point of view of the donors' blood services and GPs, this action provides the optimal reassurance to the donor. However, from a hepatology view, the clinical relevance of OBI and the need for follow-up is questioned since overdiagnosis consumes health resources and may lead to anxiety. Indeed, given the general good health of blood donors, such a response is even less likely to meet an acceptable threshold for benefit and may lead to unwarranted impacts on patients, including psychological and financial (direct and indirect, depending on the healthcare system). From a public health perspective, there is a need for clear messaging for these donors so that the notifications and any required actions are clear for the donor. Further, the importance of offering equal opportunities and the sensible use of limited resources based on risk are stressed. It was agreed that there should be clear and consistent messaging provided for blood donors with OBI during diagnosis and follow-up, and education for the staff involved in follow-up is needed to ensure the reasons behind

the referral are well understood, combined with clear guidelines and structure for healthcare providers regarding follow-up and referral. However, there may currently be a mixed message between the blood service and the hepatologist; donors cannot donate due to their infectious risk, but the infection risk is not a concern for hepatologists and the risk to individuals' health is also considered minimal. Since OBI is not without its consequences, OBI carriers who have other risk factors that increase their risk of developing liver damage should at least be given some advice. There are also practical and ethical implications of informing individuals that they have a potentially increased risk of cancer, but with no actionable recommendations; whilst transparency is important for individuals' autonomy, the ethical implications may be affected by differing healthcare systems. Individuals in low-to-middle-income countries lacking universal healthcare could be psychosocially affected by the information provided but are restricted by access and affordability to follow-up care, which may include costly diagnostic tests and monitoring strategies. Being asymptomatic, individuals may not perceive the need for follow-up care. Additionally, high-endemic countries may prioritise limited resources on public health interventions rather than individual risk communication and follow-up. In certain societies, the disclosure of an increased risk of cancer due to HBV infection may also be heavily stigmatising, potentially exacerbating psychosocial harm and adversely affecting social and economic outcomes, including access to employment or life insurance.

From the multidisciplinary discussions, there was a consensus that reviewing the information and advice provided to donors would be vital, especially surrounding the messaging on what having OBI entails for the individual. Communication with donors should be clear and culturally sensitive regarding risk notification and guidance

to accessible resources. The involvement of donors carrying OBI in research would enable a better understanding of the needs and expectations of donors who receive an OBI diagnosis. Such research would give insight into how an OBI diagnosis affects the psychological well-being of donors and whether current support mechanisms are adequate. It is expected that some donors with OBI may prefer to have the opportunity to be followed up, even though the rationale for doing so might be unclear. There exists uncertainty surrounding the infectivity of individuals with OBI and the precautions that the individual and their contacts may take. A project is already underway working with colleagues from NHSBT to interview blood donors not allowed to donate anymore due to chronic and occult HBV infection. The project aims to investigate the donors' perspectives on the services offered by NHSBT, including their thoughts, concerns, and expectations on the diagnosis and follow-up process. An understanding of donors' perspectives will help blood establishments evaluate their follow-up process to ensure that donors' views are met where necessary, and to understand donors' views of being diagnosed with this complex virus through the blood transfusion services. This project brings in PPI involvement with current and previous blood donors who shaped the interview questions, recruitment documents, and will be helping to interpret the findings.

The multidisciplinary team also identified a need to consider the oncogenic impact of OBI. Extensive and methodologically appropriate studies were necessary to investigate the real-world prevalence of OBI in cohorts of patients with chronic liver disease, especially those of cryptogenic and non-HCV-related origins. Follow-up data regarding the prognosis of OBI individuals is also needed, as well as establishing a longitudinal cohort study to ascertain the presence or absence of liver disease in OBI

blood donors. The risk of liver-related complications may also vary between different origins of OBI, e.g. occult infection following decades of HBsAg-positive infection compared to OBI following acute HBV infection or seronegative OBI individuals. Such studies may provide the necessary data to delineate individuals with OBI at risk of HCC through viral and host factors. However, blood donors are pre-selected for low frequencies of co-morbidities and low risk of infectious disease acquisition, meaning that such studies would require several decades of follow-up and should also include other groups of patients with OBI. The unrepresentative nature of donors is highlighted by the human T-lymphotropic virus (HTLV) registry study showing that blood donors with asymptomatic HTLV infection identified via blood donation were invariably asymptomatic and showed a greatly reduced risk of developing HTLV-associated myelopathy or adult T-cell leukaemia/lymphoma compared to non-donor cohorts[198]. Nonetheless, creating a global cohort of OBI donors with standardised aggregated data collection on their clinical outcomes, including complications of liver disease, would provide the necessary evidence to guide future policy for the follow-up of an OBI carrier, such as whether they should be assessed with liver function tests and an evaluation of hepatic fibrosis and steatosis.

OBI may need to be considered in WHO global hepatitis B elimination targets whilst acknowledging that the primary focus of eliminating HBsAg-positive chronic HBV infection is already challenging. There are multiple considerations to improve the OBI donor experience. Improving public health strategies and awareness campaigns to educate healthcare providers and societal communities would improve the knowledge of OBI, align the perceptions of risks among stakeholders, and reduce the stigma of HBV infection and its associated disease. Due to the lack of donor

screening for OBI in low-to-middle-income countries, there is a need for affordable and validated point-of-care reflex HBV DNA tests[199] to identify donors with OBI and prevent HBV transmission by transfusion. Given the difficulty of OBI diagnosis and the unclear association with liver disease, health initiatives for research into OBI would be crucial to achieving the WHO's goal of eliminating viral hepatitis, especially in sub-Saharan Africa, where the burden of HBV is high. There is also a need to consider the resource constraints in low-resource and high-endemic settings when managing donors with OBI. For example, adequate diagnostic tools for follow-up and a strong linkage of blood services to community-based care are needed. As with the HIV epidemic, partnerships with non-governmental, international health organisations and wealthier nations would enable access to funding, expertise, and resources[200] that may be necessary for the management of individuals with OBI. Lastly, the cost-effectiveness of OBI management strategies compared to other public health interventions could be investigated, particularly in the context of low-to-middle-income countries.

Potential biomarkers of OBI

This thesis's finding of HBV-infected donors with very low VLs highlights the existence of a potentially neglected reservoir of HBV infection in the general population, particularly when anti-HBc-positive individuals may harbour replication-competent HBV DNA in their livers despite the absence of circulating HBV DNA. Additionally, patients achieving a 'functional cure' following currently available antiviral treatment (defined as sustained undetectability of HBsAg (<0.05 IU/mL) and an undetectable or extremely low level of HBV DNA (<10 IU/mL)[201]) may still have very low VLs and potentially contribute to the growing pool of OBI carriers; limited evidence also suggests that many patients who achieve 'functional cure' also have low and persisting levels of replication-competent DNA in their livers[32]. This combined pool of OBI carriers and the ambiguity of 'functional cure' may need to be considered in elimination programs of HBV. Future therapies targeting the intrahepatic HBV DNA would be extremely beneficial in achieving a full HBV cure. Alternatively, identifying those individuals with low HBV replicative activity using alternative biomarkers may assist in targeted interventions. A major further step identified from this thesis is the challenges of current blood biomarkers of transfusion-transmitted OBI (Table 23) and the potential value of additional markers, potentially in a multivariate model, to more sensitively identify blood donors with OBI compared to anti-HBc screening, which identifies an extremely large pool of donors not currently harbouring circulating HBV DNA and could miss extremely rare cases of anti-HBc-negative OBI. Potential additional biomarkers are discussed in this final section.

Table 23.[1] Summary of the challenges and needs of blood biomarkers of transfusion-transmitted occult hepatitis B virus infection (OBI) currently utilised in diagnostic practice. HBsAg, hepatitis B surface antigen; IU/mL, international units per millilitre; anti-HBs, antibodies against surface antigen; anti-HBc, antibodies against core antigen; MP-NAT, mini-pool nucleic acid testing; ID-NAT, individual donation nucleic acid testing; cccDNA, covalently closed circular DNA; HBV, hepatitis B virus.

Biomarker	Challenges in OBI detection	Future needs
HBsAg	Extremely low levels of HBsAg expression resulting in undetectability	More sensitive assays with a limit of detection ≤0.5 IU/L to be validated in OBI donors and adopted by blood establishments
	Rare mutations in the surface gene result in the escape of detection by currently available assays	Widely available assays using multivalent polyclonal anti-HBs probes to identify donors with HBsAg mutants
	Anti-HBs/HBsAg immune complexes mask the detection of HBsAg	Investigations into the potential use of HBsAg dissociation assays in diagnostic practice
	Some donors may never develop detectable HBsAg	Screening for other biomarkers such as HBV DNA and anti-HBc
HBV DNA	Viral loads below the detection limit of MP-NAT and ID-NAT	ID-NAT testing with commercialised ultrasensitive assays
	Economically prohibitive, especially in low-and-middle-income countries	Development of point-of-care assays or novel biomarkers that are accessible for blood establishments globally
	Intermittent detection in the blood	Use of surrogate biomarkers such as anti-HBc to exclude potentially infectious donations
	Not an accurate reflection of cccDNA activity	Development of novel biomarkers that more accurately reflect intrahepatic activity

Anti-HBc	Low specificity of currently available assays results in the deferral of large numbers of donors	Re-testing reactive samples with alternative assays, development of highly specific assay, use of anti-HBc quantification
	Anti-HBc may not detect all OBI donors and may not be practical in HBV high-endemic countries	Ultrasensitive HBV DNA testing or the use of alternative biomarkers
Anti-HBs	Protective titre is not well-defined	Further investigations into the virological and immunological responses in OBI donors with differing anti-HBs levels
	Anti-HBs titre has suboptimal sensitivity and specificity to risk-stratify potentially infectious donations	Development of more sensitive biomarkers to identify potentially infectious donations

Firstly, the development and wider application of HBsAg assays that are either more sensitive for the target protein, those capable of detecting S gene mutants, and HBsAg dissociation assays will significantly affect OBI diagnosis and differentiation from patients with more active HBV infection. While their use in donor screening may identify more significant numbers of donors with detectable HBsAg, there has been evidence of acute asymptomatic HBV infections in blood donors who never develop detectable HBsAg despite persistently low HBV DNA levels and anti-HBc seroconversion[202].

An alternatively biomarker that could be investigated further is the HBV core-related antigen (HBcrAg). HBcrAg is a composite of HBeAg, HBcAg, and a truncated HBcAg coded by the pre-core/core ORF[203] (Figure 1). HBcrAg was suggested as a potential intrahepatic cccDNA transcriptional activity marker for distinguishing phases of HBsAg-positive infection[204]. Indeed, HBcrAg detection in 65% of patients

ten years after HBsAg seroconversion[205] suggests low levels of HBV protein expression in HBsAg-negative patients. However, a recent study that longitudinally sampled chronic hepatitis C patients after treatment cessation did not detect HBcrAg despite HBV DNA detectability[206]. Further evidence suggested HBcrAg as less sensitive than anti-HBc quantification for predicting cccDNA presence[122]. These findings may be confounded by assay sensitivity, where the recent development of ultrasensitive HBcrAg has shown promising results for the early detection of HBV reactivation[207]. However, the HBcrAg assay currently has limited availability in research or clinical practice, and its use in risk-stratifying OBI donors has yet to be studied. Additionally, since HBcrAg consists of multiple antigens, developing a core-specific biomarker would eliminate confounding factors such as pre-core and basal core promoter mutants that affect HBcrAg detection[208].

Pregenomic RNA (pgRNA) is another potential biomarker. PgRNA is exclusively transcribed from cccDNA[209] (Figure 1) and may indirectly measure cccDNA activity. The pgRNA intermediate functions as the mRNA for HBcAg and polymerase and is also reverse-transcribed into relaxed circular DNA for secretion from cells or for replenishment of intranuclear cccDNA[210]. Previous evidence has shown that nucleos(t)ide analogue therapy suppresses HBV DNA synthesis without equivalently reducing serum pgRNA, resulting in persistent pgRNA detectability and poor correlation with HBV DNA during treatment[211]. Another study showed that both HBcrAg and HBV RNA levels, but not HBV DNA, were shown to predict HBV reactivation[212]. These preliminary findings suggest that pgRNA levels may be a useful surrogate biomarker to reflect cccDNA transcriptional activity and possible infectivity compared to HBV DNA. Detecting pgRNA in blood via RNA PCR assays

is yet to be explored in OBI. Investigations into combined pgRNA and HBcrAg predictive value for intrahepatic activity need to be undertaken to assess these biomarkers' suitability to identify potential TTI. However, failure to detect HBV RNA by currently available assays in patients with low HBV DNA levels following treatment[212] suggests that improved sensitivities are required to detect HBV RNA in OBI donors with often very low viral loads. Using primers that target the 5' end of pgRNA in PCR assays is recommended to increase detection sensitivity and avoid RNA derived from integrations[213,214].

MicroRNA (miRNA/miR) is another potential biomarker. MiRNAs may regulate gene expression in the translational process (Figure 1) or through the degradation of HBV transcripts via imperfect base pairing[215]. Previous evidence shows that four miRNAs (Lethal-7c, miR-23b, miR-122, and miR-150) are differentially expressed in OBI compared to healthy controls[216]. MiR-122, a liver-specific miRNA, downregulates HBV gene expression and, subsequently, HBsAg and HBeAg expression[217], suggesting potential value in predicting the activity of OBI. However, with a small sample size of 11 OBI sera from a specific region and age group, further studies are necessitated to provide data on whether miRNAs could be a novel biomarker for OBI detection, where additional miRNAs such as miR-125a-5p may reduce HBsAg expression and secretion[218].

Host immune responses are critical in controlling HBV replication but remain poorly understood in OBI[17]. With the maintenance of cccDNA infecting hepatocytes in OBI[219] and reactivation of typical HBsAg-positive infection under host immunosuppression[220], it is plausible that the host immune system strongly suppresses

HBV replication (Figure 1), leading to low viral loads and lack of detectable HBsAg[221,222]. Limited evidence suggests that OBI individuals have potent, HBV-specific T-cell responses, where type 1 T helper cell (Th1) responses are quantitatively stronger than in inactive HBsAg-positive infection and are similar or higher against HBV antigens than in resolved HBV infection[223]. T-cell responses may also help distinguish seropositive and seronegative OBI, where anti-HBc positive OBI had T-cell responses comparable to resolved HBV infection, whilst seronegative OBI had minimal HBV-specific T-cell expansion, suggesting a lack of protective memory[224]. Simple cytokine stimulation and IFN-release assays have been utilised for SARS-CoV-2[225] and tuberculosis[226] detection, respectively. These assays could be adapted to detect HBsAg-specific cell-mediated responses that diminish HBsAg levels[227], where HBV-specific T-cell responses could predict periodic reactivation of OBI comparable to that characterised in other DNA viruses[228] and thus minimise the risk of TTI whilst ensuring the sustainability of the blood supply.

Human leukocyte antigen (HLA) molecules could also be useful. HLA molecules are important in epitope presentation to T-cells (Figure 1), promoting T-cell allorecognition and peptide binding, stimulating immune clearance of HBV infection[229]. A Chinese study found that the frequencies of specific HLA phenotypes associated with infection were higher in OBI than in healthy controls, whilst other phenotypes related to DNA clearance were lower[230]. Over-representation of these HLA alleles may confer enhanced T-cell responses against HBV peptides in OBI compared to HBsAg-positive infection, comparable to that recently shown in asymptomatic SARS-CoV-2 infection[231]. This over-representation may also occur in certain ethnic minorities with differing HLA frequencies[232], but validation of this in OBI donors is

necessitated. Further, homozygous compared to heterozygous HLA alleles may hamper antigen presentation[230] and impair HBV immune clearance in OBI. Specific HLA-DP single nucleotide polymorphisms (SNPs) were also associated with OBI[90]. If shown to be sensitive to OBI, identifying specific host gene variants and HLA alleles could be a valuable screening method to risk-stratify donors with enhanced immune responses against HBV replication who are at increased risk of reactivating HBV periodically.

Lastly, signalling pathways of innate immunity may be induced to varying extents in different stages of HBV infection[233] (Figure 1). Interleukin (IL)-10 is the major cytokine in limiting cell-mediated immune responses against pathogens[234], whilst IL-17 upregulates anti-apoptotic molecules in hepatocytes[235]. A study showed that IL-10 and IL-17 levels were higher in OBI than in resolved HBV infection, suggesting more suppressed immune responses and enhanced survival of virus-infected hepatocytes than in resolved infection[236]. Polymorphisms within these cytokines were suggested to hamper their expression and weaken host immune clearance of HBV infection in OBI[237]. Immune checkpoint molecules may also be somewhat affected in OBI, where the upregulation of programmed cell death protein 1 (PD-1), the most highly expressed inhibitory receptor on HBV-specific T-cells[238], resulted in T-cell dysfunction in HBsAg-positive infection[239]. Investigations into the cytokines and chemokines that may regulate periodic HBV reactivation and mild liver inflammation would provide novel perspectives on identifying donors with ongoing immunological activity in the liver, where utilisation of a multiplexed assay for screening blood donors would be able to measure hundreds of such biomarkers with small volumes of blood[240].

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Appendix 1

Details of most individual NGS protocols have been previously published and are referenced where applicable below. The protocol for PCR-A is described below. Nucleic acid extractions were performed at individual laboratories, except for three protocols for which extractions were conducted separately in the coordinating laboratory in Oxford (PCR-A, PCR-B, PCR-2).

MTG-A: The laboratory workflow is found at this reference[241], where plasma samples were extracted using the QIAsymphony DSP Virus/Pathogen Mini Kit according to the manufacturer's instructions. The bioinformatics pipeline for HBV alignment and coverage, including deduplication details, is found in this reference[242]. The bioinformatics pipeline metaMix for co-detection of viruses is found at this reference[243].

MTG-B/TAC-A/TAC-B: The laboratory and bioinformatics workflows, including the bioinformatics workflow for generating the co-detection data and read deduplication, are found in this reference[115]. For read processing, the mapped fragment length (TLEN) threshold remained at the pipeline default of 40 nucleotides. Trim settings remained at pipeline defaults, including removal of Illumina adapters. The minimum fragment length to include reads was 36 nucleotides. The HBV reference set comprises 14 full-length genomes: 12 cluster-derived consensus sequences and 2 singletons, generated from 462 HBV sequences to represent the full diversity of known HBV genomes.

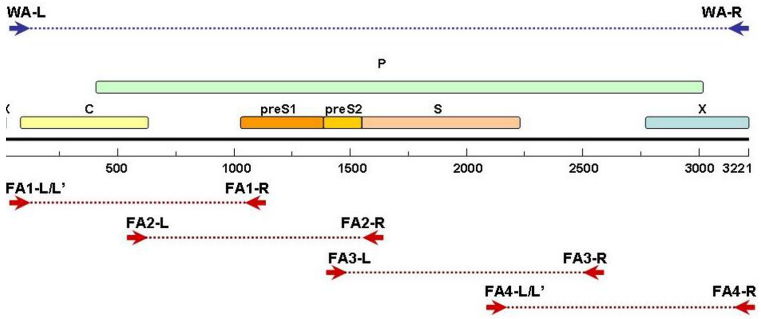
The TAC-A protocol, where a combined cDNA and gDNA protocol was used, was identical to the referenced cDNA-only protocol, except that 50% of the cDNA was

replaced with gDNA. TAC-B utilised the cDNA-only protocol, and pre-capture libraries were sequenced for the MTG-B protocol. Thus, TAC-B and MTG-B samples underwent the same sample processing before the capture step. The cDNA protocol does not include a DNase treatment step; therefore, both gDNA and cDNA are carried forward for library preparation. The TAC-A and TAC-B protocols were designed for both RNA and DNA viruses and thus were the only protocols in this study to contain a cDNA synthesis step (which may additionally sequence replication intermediates in addition to the DNA reservoir), in contrast to TAC-C, which was designed for DNA viruses only.

TAC-C: The laboratory workflow is found in this reference[149], and the bioinformatics workflow, TracesPipe, is found in this reference[244]. The minimum fragment length for raw read processing was 25 nucleotides, and deduplication was handled by samtools rmdup in TracesPipe. The TracesPipe pipeline uses FALCON, a compression-based metagenomic tool, to guide reference selection for alignment-based assembly. FALCON compares processed reads to a database of 7,555 HBV genomes and identifies the most similar genome for each sample. The database consisted of all Genbank entries labelled with a taxon ID descendant from txid10239 (viruses) as of July 11th 2019, filtered by sequence definition and length to complete HBV genomes.

PCR-A: The PCR protocol consists of a nested approach. The first PCR spans the genome, with four nested amplicons subsequently generated, 4-1018, 593-1667, 1451-2510 and 2416-3218, respectively (see diagram below). For the first PCR, the total PCR reaction volume is 25 μL, including 5 μL of DNA input per reaction. For the

second PCR, the total PCR reaction volume is 25 µl, including 1 µl first-round amplicon per reaction.



Primer sequences:

WA-L	ACTGTTCAAGCCTCCAAGCTGTGC
WA-R	AGCAAAAAGTTGCATGGTGCTGGT
FA1-L	TTTCACCTCTGCCTAATCATCTC
FA1-L'	TTT ACCTCTGCCTAATCATCTC
FA1-R	TCTTGTTCCCAAGAATATGGTG
FA2-L	GCGTCGCAGAAGATCTCAAT
FA2-R	TTGAGAGAAGTCCACCACGAG
FA3-L	CTGCTGGTGGCTCCAGTT
FA3-R	GCCTTGTAAGTTGGCGAGAA
FA4-L	GTATTGGGGGCCAAGTCTGT
FA4-L'	GTATTGGGGGCCAAATCTGT
FA4-R	AAAAAGTTGCATGGTGCTG

Equimolar pooling of amplicons from each sample was performed before library preparation with Nextera XT kit, following the manufacturer's instructions. 1 ng (5 µl of 0.2 ng/µl) pooled amplicon input was used for each library preparation reaction.

For initial bioinformatics analysis, a modified version of the ADPU (UCL Hospitals) HBV Bioinformatics Pipeline was utilised[245], incorporating deduplication but excluding the variant analysis step. This pipeline is designed for the assembly and analysis of HBV genomes from paired-end Illumina sequencing data:

1. Raw Read Processing (Tool: Trimmomatic v0.39)

Settings:

- Cores: 8
- Mode: Paired-end
- Adapter removal: ILLUMINACLIP:<adapter_file>:2:10:7
- Quality trimming: LEADING:10; TRAILING:10; SLIDINGWINDOW:4:30; MINLEN:50 [reads shorter than 50 nucleotides are removed]

2. Host Decontamination (Tool: SMALT v0.7.6)
    - Reads are aligned to a decoy reference consisting of the human genome and HBV reference sequences
    - Paired-end mapping with -x -y 0.5 -i 500 -n <cores>
    - Only non-human read pairs are retained for downstream processing
3. Decontaminated Read Extraction (Tool: samtools)
    - Extract properly paired reads from SAM file into two FASTQ files using samtools view -bhf and samtools bam2fq
    - Remove PCR duplicates and redundant reads using samtools markdup
4. De Novo Assembly (Tool: IVA (Iterative Virus Assembler) [Docker: sangerpathogens/iva])

Settings:

- --max_contigs 50
- Uses filtered paired-end reads

- Uses the same adapter and primer files as preprocessing
- Runs in a Docker container with volumes mounted for data and scripts

5. Contig Alignment and Best Reference Selection (Tool: LASTZ)
    - Contigs aligned to a reference scaffold database using LASTZ with --ambiguous=IUPAC
    - The database contains 958 full-length HBV genomes: the Refseq sequences, other references for subtypes, and the rest from Genbank, filtered by sequence length and date submitted (approximately 2020-2024; all sequences were manually inspected).
    - Best matching reference genome is selected using lastz_bestref.NO_BLAST.pl
6. Contig-to-Reference Alignment Analysis (Tool: lastz_analyser.WITH_REVCOMP.pl)
    - Determines the relationship between contigs and the best reference
    - Cutoff: 50,000 bp
    - Allows reverse complement alignment
7. Initial Consensus Generation (Tool: genome_maker2b.pl)
    - Combines mapped reads and contigs to generate a draft genome
    - Aligns reads to contigs using SMALT
    - Generates a BAM file and mpileup for consensus building
8. Consensus Polishing (First Round) (Tool: cons_mv.pl)

Settings:

- Frequency cutoff: 0.01
- Minimum depth: 100 (overall), 20 (variant), 80 (consensus)
- Sliding window: 300 bp

(Tool: N_remover_from_consensus.pl)

- Removes Ns from consensus with a cutoff of 46

9. Consensus Polishing (Second Round)
    - Same steps as above are repeated on the first polished consensus to yield a final polished consensus (consensus2)

PCR-B/PCR-2: The laboratory and bioinformatics workflows can be found at this reference[130].

PCR-1: The laboratory workflow can be found at this reference[246], and the bioinformatics workflow can be found at this reference[247], where Guppy v4.2.0 was used for base-calling. The deduplicate tool in samtools was used, but none of the reads for this study were duplicates, in agreement with the non-cluster-based sequencing performed in Nanopore.

Appendix 2

Anti-HBc and Occult HBV in blood establishments

HBV transmission via HBsAg-negative but anti-HBc-positive and HBV DNA-positive (occult HBV infection, OBI) blood products have been well-documented.

We would like to understand better how anti-HBc testing is performed worldwide, how anti-HBc-positive and OBI donors are followed up, and the prevalence of these donors. Results may influence further guidelines and recommendations.

- This **10- to 20-minute survey** aims to address the above objective and is intended for blood establishments across the globe.
- Please note that additional questions may appear depending on your answers.
- All information collected is kept confidential, and data will be presented in a pseudo-anonymised format.
- We aim to publish these findings, which may also influence future guidelines for testing.
- If you have any questions, please email michael.fu@ndm.ox.ac.uk.
- Please complete the survey by **Wednesday 6th December.**

If you use the Word version, please type your answers below each question (including typing your multiple-choice answers). Please return your completed document to michael.fu@ndm.ox.ac.uk

Demographics

1. What is the name of the blood establishment you work with? *Required*

2. How many blood donors donated at your blood establishment in 2022 (excluding fractionation-only donors)? *Required*

2.a. How many of these were new donors? *Required*

3. How many donations were collected at your blood establishment in 2022 (excluding fractionation-only donations)? *Required*

4. Please share your email address if you are happy to provide data to contribute to a publication and to receive further questions that we may have based on your answers.

4.a. Are you happy to be included as a co-author in a publication? *Required*

Yes/No

Screening for HBV

5. Under what circumstances do you screen for anti-HBc antibodies? *Required*

- anti-HBc is routinely screened for in all donations regardless of HBsAg status
- All HBsAg-positive donations are subsequently screened for anti-HBc
- All HBsAg-negative donations are subsequently screened for anti-HBc
- Only selected HBsAg-negative donations are subsequently screened for anti-HBc
- No screening for anti-HBc
- Other

If you selected Other, please specify:

If you selected 'only selected donations', how do you define selected donations?

5.c. Which assay and platform do you use for HBsAg screening?

6. When do you screen blood donations for HBV DNA? *Required*

- All blood donations are screened for HBV DNA
- All anti-HBc-positive donations are subsequently screened for HBV DNA
- Only selected anti-HBc-positive donations are subsequently screened for HBV DNA
- No screening for HBV DNA
- Other

If you selected Other, please specify:

If you selected 'only selected donations', how do you select donations for HBV DNA testing?

If you selected 'no screening for HBV DNA, is there a reason(s) for not screening HBV DNA?

6.c. Is HBV DNA screened in pools or individually?

- ○ Individually/ID-NAT
- ○ Pools of 6
- ○ Pools of 24
- ○ Other

If you selected Other, please specify:

7. Which assay is used for HBV DNA NAT/PCR? *Required*

- ○ Commercial assay
- ○ In-house assay
- ○ HBV DNA is not screened

7.a. Which commercial assay (name and company) is used?

7.b. Please provide a reference for the in-house PCR.

7.c. If you know the 95% limit of detection of the HBV DNA assay, what is this? (IU/mL) *Optional*

8. Does confirmatory testing of initially reactive anti-HBc results take place? *Required*

- ○ Yes
- ○ No
- ○ Anti-HBc is not screened

8.a. How do you confirm anti-HBc-positive results?

9. Pathogen inactivation is performed on blood components at my blood establishment (plasma and red cells). *Required*

- Yes, pathogen inactivation is applied to all components.
- Yes, pathogen inactivation is applied to some components.
- No, pathogen inactivation is not applied.

9.a. When is pathogen inactivation applied and what sort of pathogen inactivation is performed?

9.b. To which components is pathogen inactivation applied?

Data on occult hepatitis B virus infections

10. How many new donors/donations were anti-HBc positive (regardless of other HBV markers) in your blood establishment in 2022? Type N/A if anti-HBc is not screened. *Required*

10.a. How many repeat donors/donations were anti-HBc positive (regardless of other HBV markers) in your blood establishment in 2022? Type N/A if anti-HBc is not screened. *Required*

11. What is the number of (a) donors, and (b) donations with OBI (HBsAg-negative, HBV DNA positive) in your blood establishment in 2022? Type N/A if HBV DNA is not tested in any

HBsAg-negative donors. *Required*

11.a. What is the range of viral loads for those OBI donors in IU/mL (optional)? *Optional*

12. Have there been documented and confirmed HBV transmission from blood donors with OBI at your blood establishment? *Required*

- ○ There have been documented and confirmed transmissions.
- ○ There have been documented but unconfirmed transmissions.
- ○ No, there have not been documented transmissions.
- ○ I am not sure.
- ○ Not applicable e.g., transmission are not allowed to be investigated

12.a. How many confirmed transmissions from OBI blood donors have there been in the last 5 years? Type 'not sure' if unsure.

12.b. How many documented but unconfirmed transmissions have there been in the last 5 years? Type 'not sure' if unknown or unsure.

Follow-up of donors

13. Are HBsAg-negative, HBV DNA negative donors who test anti-HBc initially reactive but unconfirmed (indeterminate) allowed to donate? Excluding fractionation-only donors.
Required

- ☐ All of these donors are allowed to donate
- ☐ Allowed but with certain conditions
- ☐ No

13.a. Under what condition(s) are solely anti-HBc-positive donations accepted into the blood supply?

- ○ If anti-HBs levels are above a certain limit
- ○ Other

13.a.i. If you selected Other, please specify:

13.a.ii. If you selected anti-HBs limit, what is the anti-HBs limit at which solely anti-HBc positive donations are accepted?

13.b. For donors not allowed to donate, is deferral temporary or permanent?

- ○ Temporary
- ○ Permanent
- ○ Other

13.b.i. If you selected Other, please specify:

14. Are HBsAg-negative, HBV DNA-negative donors who test anti-HBc confirmed allowed to donate? Excluding fractionation-only donors. *Required*

- [ ] All of these donors are allowed to donate
- [ ] Allowed but with certain conditions
- [ ] No

14.a. Under what condition(s) are solely anti-HBc-positive donations accepted into the blood supply?

- ( ) If anti-HBs levels are above a certain limit
- ( ) Same answer as the previous question
- ( ) Other

14.a.i. If you selected Other, please specify:

14.a.ii. What is the anti-HBs limit at which solely anti-HBc positive donations are accepted?

14.b. For donors not allowed to donate, is deferral temporary or permanent?

- ( ) Temporary
- ( ) Permanent
- ( ) Other

14.b.i. If you selected Other, please specify:

15. How are HBsAg-negative and anti-HBc positive donors without HBV DNA followed up?
Required

- ☐ My blood establishment is not responsible for follow-up
- ☐ Referral to microbiology/infectious diseases
- ☐ Referral to hepatology
- ☐ Results provided to the donor and to their GP/family doctor
- ☐ Other - please specify below
- ☐ No follow-up

15.a. Other follow-up(s)

16. How are OBI (HBsAg-negative, DNA-positive and not in the acute phase) donors followed up?
Required

- ☐ My blood establishment is not responsible for follow-up
- ☐ Referral to microbiology/infectious diseases
- ☐ Referral to hepatology
- ☐ Results provided to the donor and to their GP/family doctor
- ☐ Deferral of positive donation but donor allowed to come back
- ☐ Permanent deferral of donor
- ☐ Other - please specify below
- ☐ No follow-up

16.a. Other follow-up(s)

17. Please type below ifyou have further comments regarding the entire survey

Thank you very much for participating in this survey. Please return this Word document to michael.fu@ndm.ox.ac.uk, with any questions that you may have.