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RESEARCH ARTICLE

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Ultrasensitive PCR system for HBV DNA detection: Risk stratification for occult hepatitis B virus infection in English blood donors

Michael X. Fu[1] | Peter Simmonds[1] | Julien Andreani[1,2] | Hatice Baklan[3] | Mhairi Webster[3] | Romisa Asadi[1] | Tanya Golubchik[1,4] | Judith Breuer[5] | Samreen Ijaz[6] | Ines Ushiro-Lumb[3] | Su Brailsford[3] | William L. Irving[7] | Monique Andersson[8,9] | Heli Harvala[3,5,8]

[1]Nuffield Department of Medicine, University of Oxford, Oxford, UK

[2]Centre Hospitalier Universitaire Grenoble-Alpes, Grenoble, France

[3]Microbiology Services, NHS Blood and Transplant, Colindale, UK

[4]Sydney Infectious Diseases Institute, Faculty of Medicine and Health, University of Sydney, Sydney, Australia

[5]Division of Infection and Immunity, University College London, London, UK

[6]Virus Reference Department, Blood Borne Virus Unit, UK Health Security Agency, London, UK

[7]NIHR Nottingham Biomedical Research Centre, Nottingham University Hospitals NHS Trust, University of Nottingham, Nottingham, UK

[8]Radcliffe Department of Medicine, University of Oxford, Oxford, UK

[9]Department of Microbiology, Oxford University Hospitals NHS Foundation Trust, Oxford, UK

Correspondence
Heli Harvala, Radcliffe Department of Medicine, University of Oxford, Oxford, UK.
Email: heli.harvala@nhsbt.nhs.uk

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Abstract

Occult hepatitis B (HBV) infection (OBI), characterized by low viral loads, accounts for much of the risk of HBV transfusion-transmitted infection. With anticore antibodies (anti-HBc) screening introduced in England, the imperative to identify OBI donors has increased. We aimed to develop an ultra-sensitive PCR system and investigate risk factors for HBV DNA presence in blood donations. Seven extraction methods and three PCR assays were compared. The optimal system was sought to determine HBV DNA presence in anti-HBc-positive donations. Predictors of DNA positivity were subsequently investigated. Extraction from 5 mL of plasma increased sample representation and resulted in HBV DNA detection in low viral load samples (~0.5 IU/mL). Screening of 487 763 donations in 2022 identified two OBI donors and 2042 anti-HBc-positive donors, 412 of the latter with anti-HBs < 100 mIU/mL. Testing of 134 anti-HBc-positive donations utilizing the 5 mL extraction method identified two further HBV DNA-positive donations. Higher anti-HBc titer and anti-HBs negativity were significant predictors of DNA detectability in anti-HBc-positive donations. An ultrasensitive PCR assay identified potentially infectious donations increasing HBV DNA detection in anti-HBc-positive donors from 0.5% to 1.9%. Anti-HBc titers may further complement the risk stratification for DNA positivity in anti-HBc screening and minimize unnecessary donor deferral.

KEYWORDS
anti-HBc screening, HBV-ID-NAT, hepatitis B virus, occult HBV infection, predictive markers

Institution where the work was performed: Nuffield Department of Medicine, University of Oxford, Oxford, UK.

1 | INTRODUCTION

Hepatitis B virus (HBV) infection constitutes a major global health burden, with an estimated 296 million people chronically infected in 2019.[1] This DNA virus is highly endemic in geographical areas such as sub-Saharan Africa, South-East Asia, China, and South America.[2] HBV infection can be acquired through various routes, including vertical transmission, sexual contact, and unsafe injections.[2] HBV is also the most prevalent transfusion-transmitted viral infection despite screening being implemented.[3] The residual risk of transmission following routine screening is associated with hepatitis B surface antigen (HBsAg)-negative preseroconversion "window period" donations in early acute infection and donations during later stages of infection with the presence of HBV DNA but the absence of detectable HBsAg, known as occult HBV infection (OBI).[4] OBI is an under-recognized clinical entity. Increasing evidence suggests that OBI accelerates cirrhosis and is a risk factor for hepatocellular carcinoma.[5,6] Moreover, an estimated 8%–29% of recipients of blood products from donors with OBI become HBV-infected,[7,8] with a risk of fulminant acute hepatitis in the immunocompromised recipient or those with underlying liver disease.[9]

HBV screening of blood donations in England includes testing for HBsAg in individual donations and for HBV DNA by nucleic acid testing (NAT) in pools of 24 donations.[7] This screening strategy has not prevented transfusion-transmitted HBV infections from donors with OBI. OBI donors are usually characterized by the presence of anti-core antibodies (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/mL.[8] With individual NAT screening, this requires a PCR 95% limit of detection (LOD) of 0.150 IU/mL to avoid potential transmission via components containing 20 mL of plasma.[8] 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 alternatively introduced to defer potentially infectious donations from the blood supply.

Following recommendations from the Standing Advisory Committee on the Safety of Blood, Tissues, and Organs (SaBTO),[10] UK blood services introduced routine anti-HBc screening in May 2022 to further reduce the risk of OBI donations reaching the blood supply. 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.

We aimed to develop an ultrasensitive PCR system for HBV DNA detection, through investigations of the clinical performance of different extraction and amplification methods. HBsAg-negative, anti-HBc-positive blood donations with low anti-HBs levels (<100 mIU/mL), more likely to be associated with infectivity due to low levels or absence of neutralizing antibodies,[8] were subsequently re-tested for HBV DNA using the most sensitive assay. We also investigated risk factors in these blood donors potentially predictive of detectable DNA.

2 | MATERIALS AND METHODS

2.1 | 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 Roche Large Volume Extraction Kit (Rochel). 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 NHS Blood and Transplant (NHSBT) routine screening (Table 1) and utilized for PCR system development. The anti-HBc screening strategy at NHSBT and the ethics statement are detailed in the Supporting Information. To determine the frequency of HBV DNA positivity in anti-HBc-positive donors, the first available 134 plasma packs that tested anti-HBc-positive, DNA-negative by individual NAT and anti-HBs <100 mIU/mL between May 30, 2022 and December 31, 2022 were received from NHSBT.

2.2 | Testing data

Anonymised virological data (HBV DNA VL, genotype, anti-HBs titer 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 134 donations assayed in the current study. These were obtained from NHSBT.

2.3 | DNA extraction 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" of the main text) was added to 200 µL Buffer AL, ethanol was increased from 200 to 230 µL and 60 µL buffer AE was used instead of 50 µL. Moreover, novel modifications to the Roche2.5 protocol to utilize larger extraction volumes[11] (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 min), 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

TABLE 1 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).

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
Sample 1	+	D2	D2	533	919
Sample 2	+	D	Not done[a]	3	Not detected
Sample 3	+	A	A2	2715	2960
Sample 4 (OBI)	–	A3	Not done[a]	3	Not detected
Sample 5	+	B4	B4	55	56.9
Sample 6	+	E	E	42	240
Sample 7	+	A	Not done[a]	25	Not detected
Sample 8	+	D1	D1	228	482
Sample 9[b]	+	E	Not done[c]	1871	3520
Sample 10	+	D3	D	22	18.7
Sample 11	+	A2	A2	7115	7980
Sample 12	+	C2	C2	670	947
Sample 13[b]	+	A2	Not done[c]	475 689 329	102 000 000
Sample 14	+	A	A	599	712
Sample 15	+	E	E	141	379
Sample 16 (OBI)	–	A	Not done[a]	25	5.07
Sample 17	+	D3	D3	619	661
Sample 18	+	B	B	4 873 759	18 800
Sample 19 (OBI)	–	C5	Not done[a]	27	49.9
Sample 20	+	C5	Not done[c]	48 242 946	1 720 000
Sample 21	+	C5	Not done[c]	41	87.5
Sample 22[b]	+	D1	D1	3881	4390
Sample 23	+	A	A2	53	190
Sample 24	+	A1	Not done[a]	38	99.9
Sample 25	+	D	Not done[c]	550	2860
Sample 26 (OBI)	–	A	Not done[a]	24	13.9

Abbreviation: OBI, occult hepatitis B virus infection.

[a]Genotyping at UKHSA not done for six samples as viral load was undetectable or below the level of quantification.

[b]The three selected samples for PCR assay development, where Sample 13 was further utilized as an internal control calibrated against the HBV DNA 4th International Standard.

[c]Genotyping at UKHSA was not done for five samples due to the COVID-19 pandemic.

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.

2.4 | Real-time PCR

Three published PCR methods for HBV DNA amplification were selected for comparison[12–14] (PCR-A to C; Table 2). First, 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 (Supporting Information: Figure S1). Second, the sensitivity of these three PCR methods was assessed by testing serial dilutions of three samples selected from the panel and standardized against the International Standard. The real-time PCR protocol is detailed in the Supporting Information. The sensitivities of PCR methods were evaluated, and the best method

TABLE 2 Sequences of the three primer/probe sets, details about the source of sequences and reported sensitivities.

Primer/probe set	Sense primer	Antisense primer	Probe	Source and modifications
PCR-A	TCYTGGCCAAA	GRTARTCCAGAA	FAM-CTGGATGTGT	Previous 50% LOD 8.4 IU/mL.[14] The probe position was altered to change nonmatching ends, and the antisense primer was modified to a 90% consensus sequence of all HBV subgenotypes.
	ATTCGCAGTCCC	GAACCAAYAAGAAG	CTGCGGCGTTTTATC-BHQ1	
PCR-B	GTGTCTGCGGC	GACAAACGGGCA	FAM-CCTCTKCATCCT	Previous 95% LOD 24.4 IU/mL[13]
	GTTTTATCA	ACATACCTT	GCTGCTATGCCTCATC-TAMRA	
PCR-C	CAACCTCCAAT	ATATGATAAAACG	FAM-TCCTCCAATTTG	Previous 95% LOD 2 IU/mL.[11] Use of BHQ1 quencher instead of TAMRA
	CACTCACCAAC	CCGCAGACAC	TCCTGGTTATCGCT-BHQ1	

Note: Modifications made to PCR-A are detailed in the table.
Abbreviation: LOD, limit of detection.

was used for testing anti-HBc-positive donations. Multiple replicates of sequential 1:2 dilutions of the International Standard from 45.36 to 0.09 IU 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.

2.5 | 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 (Supporting Information: Table S1).[15] These protocols are detailed in the Supporting Information.

2.6 | Statistical analyses

Based on linear regression, average cycle threshold (C_t) 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]. C_t 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. All analyses were performed with GraphPad Prism (v9.5.1, LLC), except Probit analysis on SPSS (v28.0.0.0, IBM). Statistical significance was set at $p \leq 0.05$.

3 | RESULTS

3.1 | Characterization of HBV plasma panel

Twenty-six plasma samples were utilized to assess the sensitivity and specificity of a range of PCR assays for HBV DNA (Table 1). To confirm genotype assignments and further characterize 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 (Supporting Information: Figure S2). These assigned genotypes matched the available genotypes determined by the UK Health Security Agency (UKHSA) (Table 1).

3.2 | 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 2). 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 hybridization affinity (Supporting Information: Figure S1). 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. C_t 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 1).

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. C_t values at each dilution were comparable between assays, except for consistently lower C_t 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; C_t values of each sample tested undiluted were generally comparable between three PCR assays (Figure 2A). When comparing each C_t value to the mean C_t (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 C_t 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 C_t values than the mean for genotype A samples (Figure 2B). This partially enhanced amplification efficiency and increased sensitivity shown earlier justified the utilization of PCR-C for the remaining measurements.

3.3 | Comparison of nucleic acid 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 3). 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

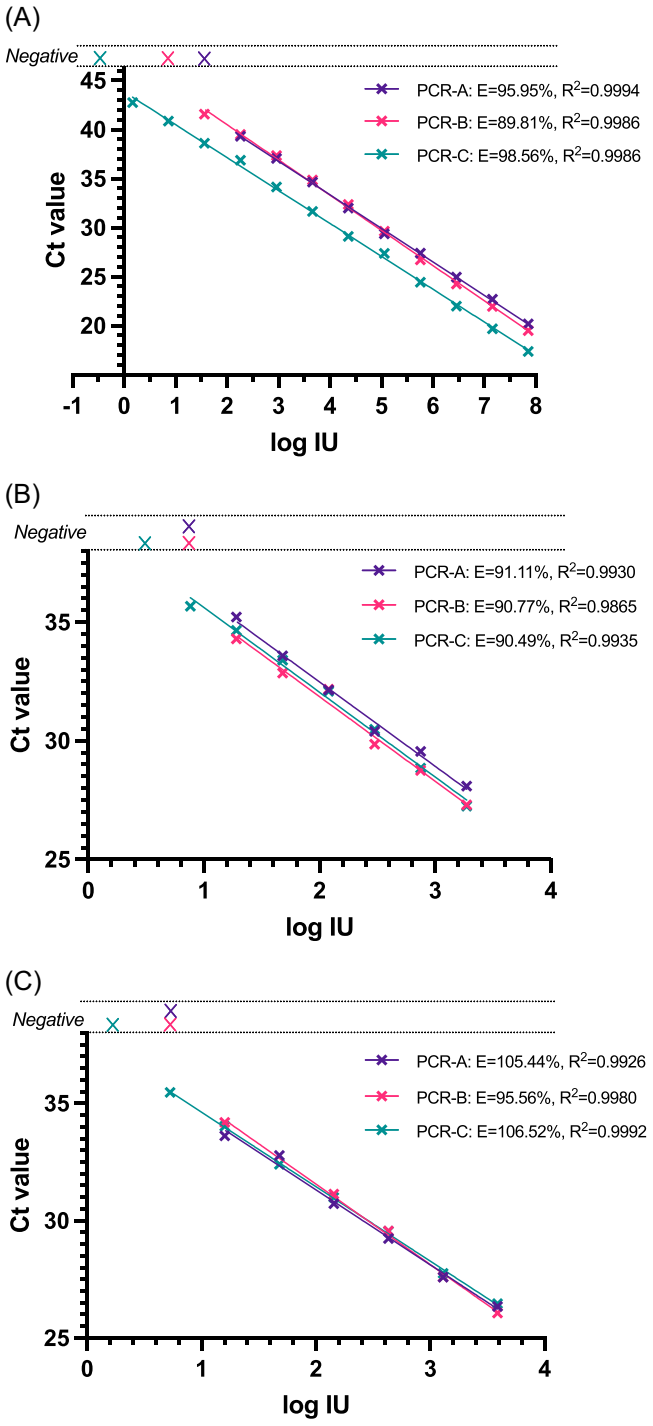


FIGURE 1 Comparison of C_t values obtained between the PCR primer/probe sets using serial dilutions of three samples of different genotypes: (A) Serial 1:5 dilutions of Sample 13 [genotype A2; has no base mismatches with PCR-C], (B) Serial 1:2.5 dilutions of Sample 9 [genotype E; sample with base mismatches 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.

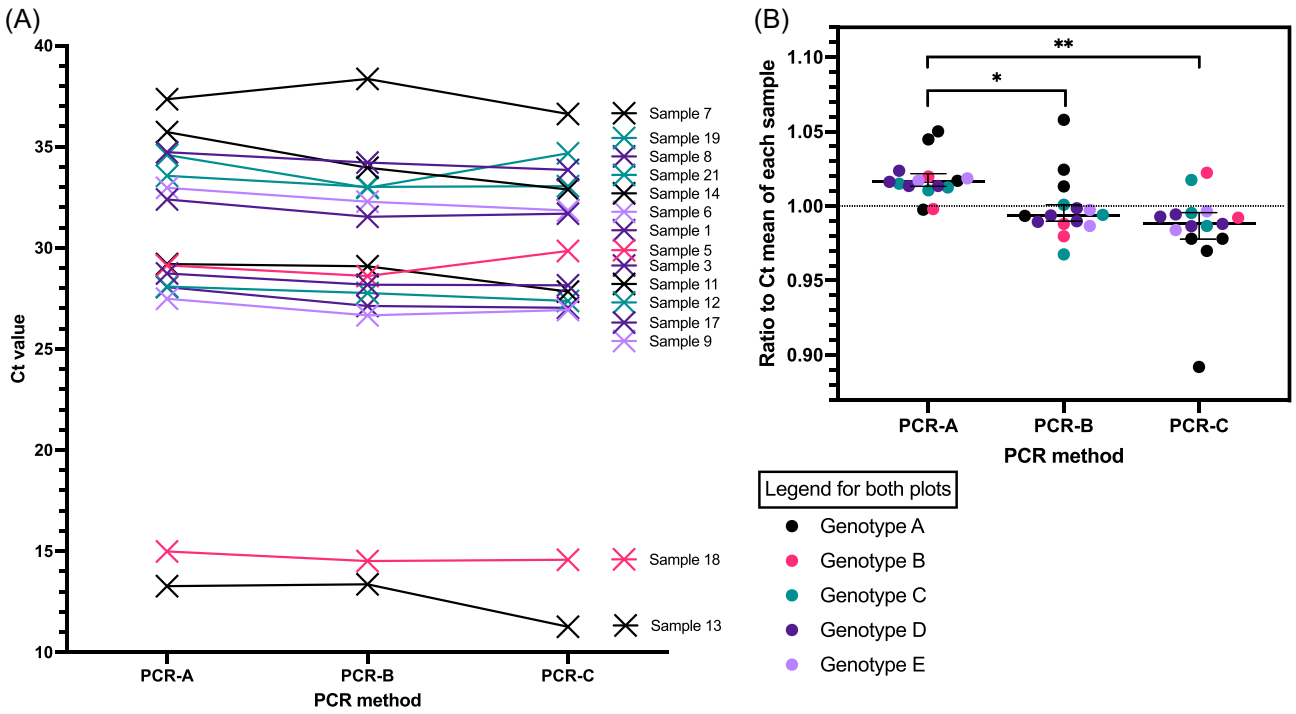


FIGURE 2 (A) Comparison of C_t values in the PCR methods; (B) Comparison of PCR methods of the ratio of C_t values for samples to the mean across all methods. Each genotype is categorized in a different color for both plots, as shown in the legend. The median and interquartile ranges for each method are displayed, with the continuous dotted line showing the C_t mean ratio of each sample as 1.0. $^*p \leq 0.05$ and $^{**}p \leq 0.01$ assessed by post hoc Dunn's test via Friedman test.

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 3) 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.

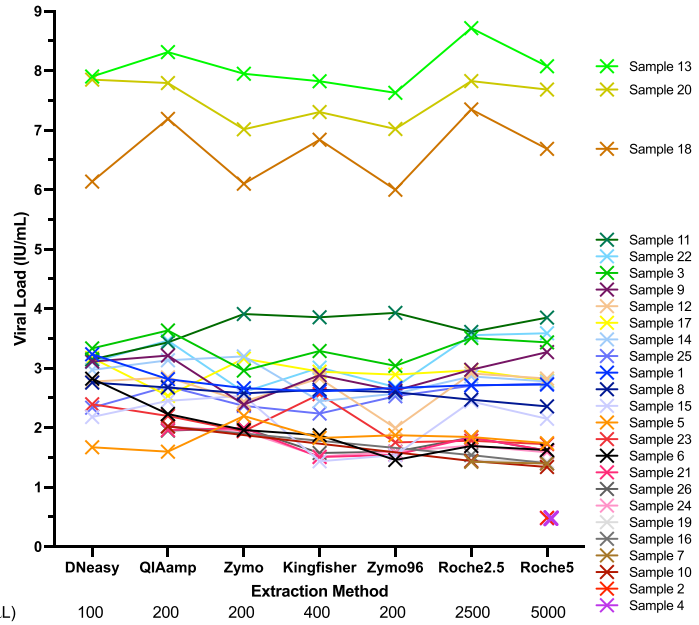
HBV recovery efficiencies were compared for the 17 control samples detected by all methods to further compare the extraction methods. The log-transformed VLs for each sample detected with each extraction method were expressed as ratios to log-transformed VLs across all methods for that sample (Supporting Information: Figure S3). 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.

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 (Supporting Information: Figure S4). The necessity to increase sample representation to detect OBI samples characterized 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.

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: 0.085 IU/mL, 0.170 IU/mL), respectively (Figure 4). 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).

3.4 | 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



	DNeasy	QIAamp	Zymo	Kingfisher	Zymo96	Roche2.5	Roche5
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

FIGURE 3 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.

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 mIU/mL by a commercial PCR assay (Figure 5). Testing of the first available 134 anti-HBc-positive donations with anti-HBs < 100 mIU/mL (where the plasma pack was available) identified two 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 (Supporting Information: Figure S2). The other 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.

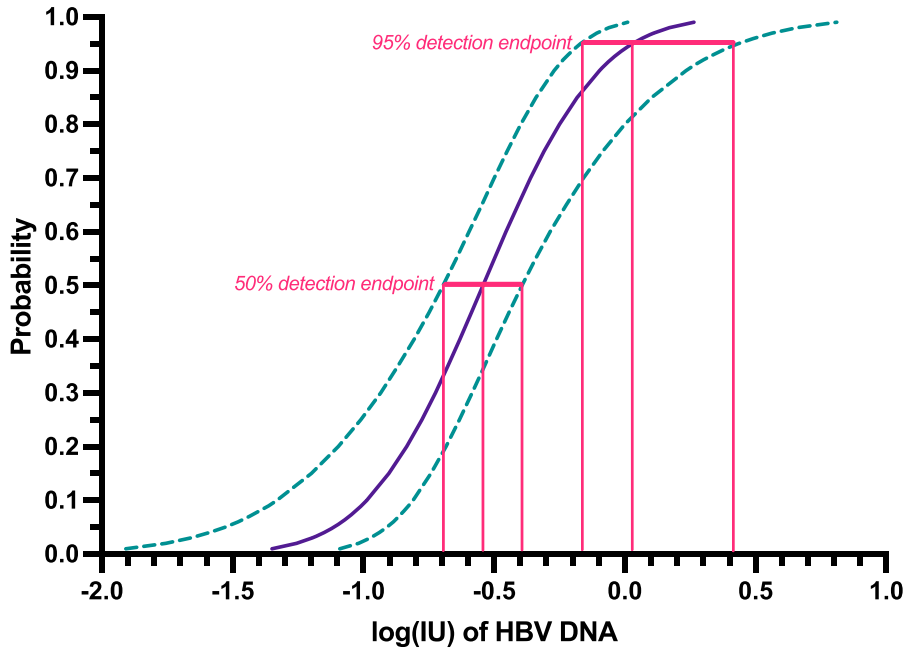
3.5 | Risk stratification

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 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 toward more anti-HBe-negatives in the DNA-positive group compared to the DNA-negative group ($p = 0.057$) (Supporting Information: Table S2).

DNA-positive donors had significantly lower median anti-HBs titers (9.4 [1.9–38.0] mIU/mL) than DNA-negative donors (21.2 [6.5–56.7] mIU/mL; $p = 0.040$; Figure 6A). 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 6B). 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 mIU/mL 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 6C).

4 | DISCUSSION

This study 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



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

FIGURE 4 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 45.36–0.09 IU used for Probit analysis are shown below the plot. HBV, hepatitis B.

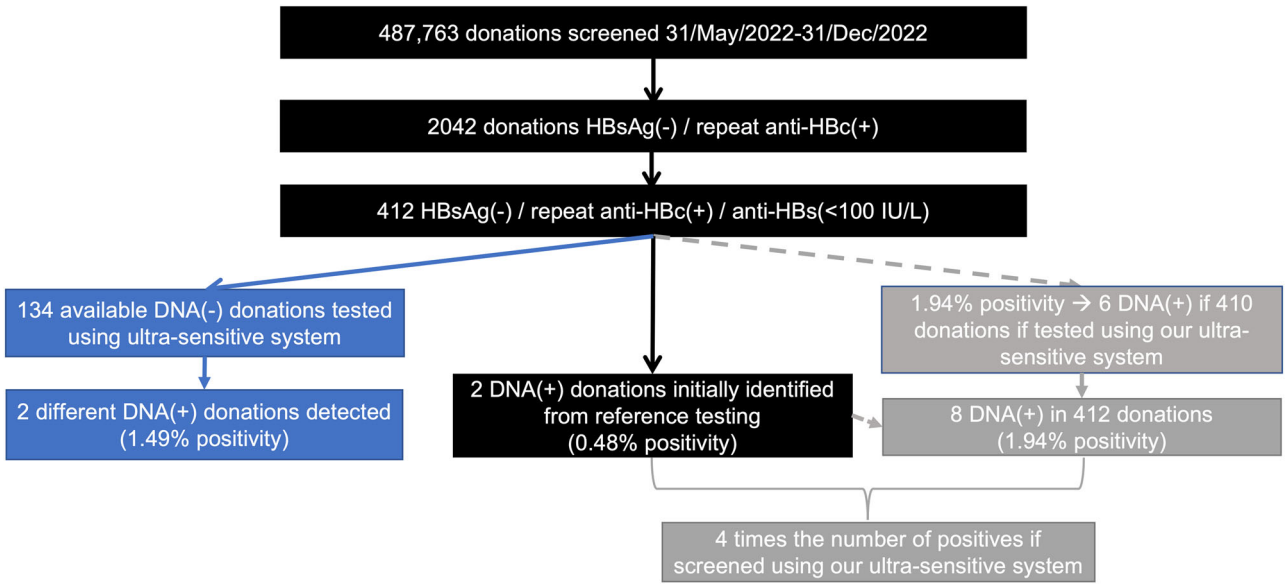
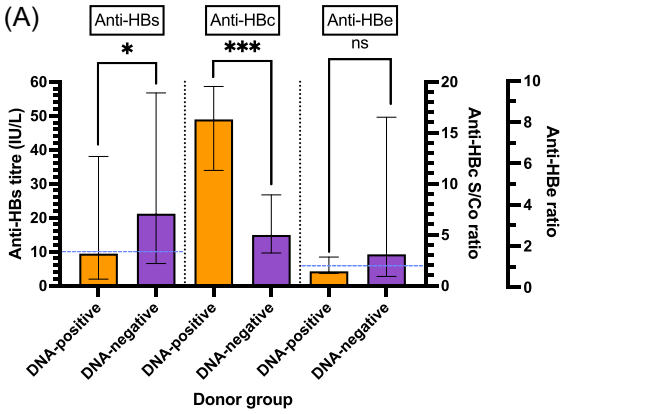
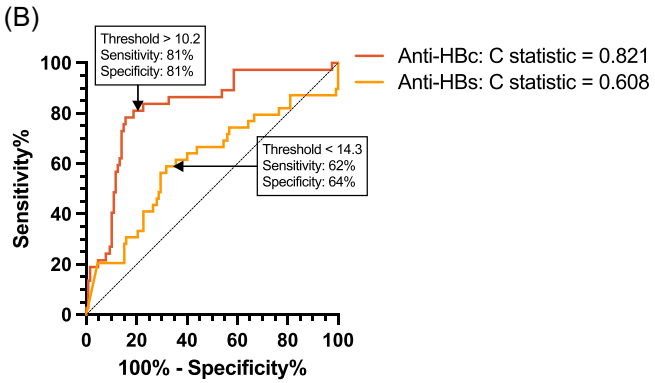


FIGURE 5 Screening and reference lab testing pathway for NHSBT donations since the introduction of anti-HBc screening in May 2022 (black boxes). One hundred and thirty-four of 412 confirmed anti-HBc-positive donations with low anti-HBs levels (<100 mIU/mL) were assayed by ultrasensitive PCR (blue boxes). Extrapolation of HBV DNA detection rate to all 412 HBsAg-negative, anti-HBc-positive, and anti-HBs < 100 mIU/mL donations is shown in gray boxes. HBV, hepatitis 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

FIGURE 6 (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 mIU/mL) and anti-HBe (1) where values below are considered negative. $^{*}p \le 0.05$, $^{***}p \le 0.001$ assessed by Mann–Whitney *U* tests, and ns denotes nonsignificance; (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.

added to the PCR) contributes proportionally to assay sensitivity. Applying this assay, we detected further 1.5% samples from anti-HBc-positive, HBsAg-negative donors that were HBV DNA-positive but negative on standard reference testing. This supports the potential utilization 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, we demonstrated that larger extraction volumes translate to improved detection of HBV DNA, consistent with previous studies using in-house[11] and commercial assays.[11,16,17] 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.[11] With a wider range of HBV VLs, the current study 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.

Genome sequences of HBV human variants may differ by up to 10%–13% from each other[15] 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,[12] there were base mismatches in PCR-C with certain HBV genotypes at two sites in the probe (Supporting Information: Figure S2). 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.[11] Our results suggest that the mismatches may not have substantially destabilized 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[18] and influenza[19] that suggested that any effects on PCR product yield were dependent on the positions and nature of the mismatches.

Our assay's 95% LOD of 0.450 IU/mL is 10-fold lower than commercial assays utilized 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, our assay detects more than twofold fewer HBV DNA IUs than the Grifols commercial assay (Supporting Information: Table S3). Testing of our optimal system on 134 plasma donations identified two further HBV DNA-positive donations that were undetectable with these commercial assays. 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.[9] Given the recently proposed lower limit of VLs associated with infectivity (0.15 IU/mL),[8] the 50% detection endpoint of the current study's assay (0.121 IU/mL) would hypothetically detect and subsequently prevent cases of OBI transmission characterized by extremely low VLs.

The DNA positivity rate in the current study of 8/412 (1.9%, CI [0.8–3.8]) (Figure 5) 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[20] 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[8] and more likely to contain HBV DNA. This suggests a lower but still significant proportion of OBI donors in England compared to Switzerland, which follows the lower anti-HBc positivity rate amongst all blood donors.[7,20] Considering the DNA positivity rate in our study cohort, an 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 to 2018 and an anti-HBc prevalence of 0.30%.[7] Our assay would detect four-fold more DNA positives than expected from the current screening strategy (Figure 5). However, larger volume extractions with 5 mL are economically costly and labor-intensive for blood services compared to current automated testing. We suggest that it may be more practical and economical to screen ID-NAT-negative donations with high anti-HBc titers using our ultrasensitive system to increase the likelihood of detecting DNA-positive donations of extremely low VLs. A mitigation strategy could also be utilized, 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.[21]

Our study provides evidence that there is a higher risk subgroup of anti-HBc donors for OBI, irrespective of intermittent viraemia that some might show.[22,23] Anti-HBc titers in sera may reflect the response to HBV core antigens generated and released into the bloodstream by higher levels of intrahepatic replication-competent covalently closed circular DNA (cccDNA), where a previous study found an association between anti-HBc IgG values with the detection of cccDNA.[24] However, that study found no association in anti-HBc titer with the presence of viraemia,[24] 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 study 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.[24] Additionally, the previous study's small population of six DNA-positive donors[24] decreases validity compared to the 42 in the current study. 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,[25] 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.[26]

Our findings support the use of anti-HBc and anti-HBs titers as serological biomarkers complementary to NAT to better define noninfectious donors without DNA, with an anti-HBc titer cut-off of 10.2 (Architect anti-HBc II) that could identify OBI donors with detectable DNA who have increased risks of hepatocellular carcinoma.[6] This supports previous suggestions in immunocompromised populations that a combination of high anti-HBc titer and low anti-HBs titer would identify those at increased risk of HBV reactivation.[27] The use of these biomarkers may help minimize 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,[28] and is especially important for HBV-endemic countries where the prevalence of anti-HBc is high and universal anti-HBc screening would be unsustainable.[25] However, while anti-HBc and anti-HBs titers were predictive of viraemia detection on ROC analysis (Figure 6B), the association was imperfect (<100% specificity) and there were indeed HBV DNA-positive samples among samples with low anti-HBc levels. We have plans to investigate the predictive values of other host response markers to differentiate DNA-positive and DNA-negative donors.

A limitation of the current study was that 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.

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 no 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 titers) that may enable more effective risk stratification for infectivity, perhaps enabling the current extensive deferral of donors to be reversed.

AUTHOR CONTRIBUTIONS

Heli Harvala: Conceptualization; methodology; resources and data curation; data analysis; writing—critical revision; supervision. **Peter Simmonds**: Conceptualization; methodology; data analysis; writing—critical revision; supervision. **Monique Andersson**: Methodology; data analysis; writing—critical revision; supervision. **Michael X. Fu**: Methodology; investigation; data analysis; Writing—original draft. **William L. Irving**: Methodology; data analysis; writing—critical revision. **Su Brailsford**: Methodology; data analysis; writing—critical revision. **Ines Ushiro-Lumb**: Methodology; data analysis; writing—critical revision. **Samreen Ijaz**: Methodology; data analysis. **Hatice Baklan**: Resources and data curation. **Mhairi Webster**: Resources and data curation. **Julien Andreani**: Investigation; writing—critical revision. **Romisa Asadi**: Investigation. **Tanya Golubchik**: Data analysis. **Judith Breuer**: Data analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by the Blood Supply Clinical Audit, Risk and Effectiveness Committee of NHSBT. All methods were performed following the relevant guidelines and regulations under the Declaration of Helsinki. Informed consent was obtained at the time of donation.

ORCID

Michael X. Fu http://orcid.org/0000-0002-3108-7917
Peter Simmonds http://orcid.org/0000-0002-7964-4700

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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