Received: 4 April 2024 | Accepted: 21 June 2024

DOI: 10.1002/jmv.29774

RESEARCH ARTICLE

JOURNAL OF MEDICAL VIROLOGY WILEY

Reconstruction of the historic time course of blood-borne virus contamination of clotting factor concentrates, 1974–1992

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Funding information

National Institutes for Health Research, Grant/Award Number: NIHR203338

Abstract

Factor VIII and IX clotting factor concentrates manufactured from pooled plasma have been identified as potent sources of virus infection in persons with hemophilia (PWHs) in the 1970s and 1980s. To investigate the range and diversity of viruses over this period, we analysed 24 clotting factor concentrates for several blood-borne viruses. Nucleic acid was extracted from 14 commercially produced clotting factors and 10 from nonremunerated donors, preserved in lyophilized form (expiry dates: 1974–1992). Clotting factors were tested by commercial and in-house quantitative PCRs for blood-borne viruses hepatitis A, B, C and E viruses (HAV, HBV, HCV, HEV), HIV- types 1/2, parvoviruses B19V and PARV4, and human pegiviruses types 1 and 2 (HPgV-1,-2). HCV and HPgV-1 were the most frequently detected viruses (both 14/24 tested) primarily in commercial clotting factors, with frequently extremely high viral loads in the late 1970s–1985 and a diverse range of HCV genotypes. Detection frequencies sharply declined following introduction of virus inactivation. HIV-1, HBV, and HAV were less frequently detected (3/24, 1/24, and 1/24 respectively); none were positive for HEV. Contrastingly, B19V and PARV4

were detected throughout the study period, even after introduction of dry heat treatment, consistent with ongoing documented transmission to PWHs into the early 1990s. While hemophilia treatment is now largely based on recombinant factor VIII/IX in the UK and elsewhere, the comprehensive screen of historical plasma-derived clotting factors reveals extensive exposure of PWHs to blood-borne viruses throughout 1970s-early 1990s, and the epidemiological and manufacturing parameters that influenced clotting factor contamination.

KEYWORDS

clotting factor, Factor IX, Factor VIII, hemophilia, hepatitis A virus, hepatitis B virus, HIV-1, human pegivirus, PARV4, parvovirus B19

1 | INTRODUCTION

Hemophilia A and B are genetic diseases in which lack of factor VIII (FVIII) or factor IX (FIX) production can lead to severe bleeding disorders. Untreated hemophilia is a disabling and potentially fatal condition, and there has been substantial investment in its medical treatment, starting sequentially from the 1950s with replacement therapy by plasma transfusion, the use of cryoprecipitate that provides FVIII in a more concentrated form and the subsequent introduction of lyophilized FVIII and FIX-enriched preparations from plasma fractionation methods developed in the early 1970s (reviewed in Mannucci[1]). These have now been progressively replaced by synthetically produced recombinant proteins in developed countries during the 1990s, although plasma-derived concentrates are still primarily used in low- to middle-income countries.

Throughout this period of treatment development, the virus transmission risk from pooled plasma-derived clotting factor concentrates was increasingly recognized.[2] In particular, the practice of generating products from often large pools of plasma derived from multiple donors, appeared to exacerbate the risk of transmission of hepatitis B virus (HBV) infections. Persons with hemophilia (PWHs) were additionally at high risk of developing a chronic hepatitis unrelated to HBV or hepatitis A virus (HAV) infections, termed non-A, non-B hepatitis (NANBH) and subsequently shown to result from infection with hepatitis C virus.[3,4] It was additionally recognized from the early 1980s that PWHs in the United States were at risk for developing AIDS,[5] subsequently linked to the appearance of human immunodeficiency type 1 (HIV-1) antibodies.[6] HIV-1 infection was widely documented in PWHs in other Western countries, particularly among users of US-sourced factor VIII and IX concentrates.[7] Plasma-derived clotting factors may additionally contain and transmit a range of other blood-borne viruses, including parvovirus B19V, the distantly related parvovirus, PARV4, hepatitis A virus (HAV), and human pegiviruses (HPgVs).[8–12] The contribution of the latter viruses to blood product safety is not well defined. B19V and HAV infections occur widely in the community with respiratory and enteric routes of transmission and are typically mild or nonpathogenic resolving infections in immunocompetent individuals. HPgV type 1 (HPgV-1) is similarly widely distributed with measurable frequencies of active viraemia from persistent infections in blood donors and the wider populations without known disease associations,[13] although a recent systematic reviews reports an association with development of lymphoma.[14] PARV4 and HPgV-2 infections are much less common in donors and have been detected primarily in association with injecting use and concurrent HCV infection; whether they exacerbate hepatitis or cause other systemic disease is unknown.[9,15,16]

The discovery of widespread HIV-1 infection in PWHs in the early 1980s led to urgent measures to prevent further transmission via blood products. Virus inactivation and removal methods were adopted by manufacturers from 1984 to 1995,[17] including the use of solvent/detergent treatment (largely effective only against enveloped viruses) through to extreme dry heat (≥90 C) and from the 1990s, viral exclusion methods such as nanofiltration and affinity purification of FVIII/FIX with monoclonal antibodies. The use of viral inactivation methods was highly effective against HIV and HCV although instances of B19V, HAV, and PARV4 transmission continued to occur,[9–11] reflecting their thermal stability. Donor selection was enhanced to defer donors with risk factors and symptoms of HIV infection/AIDS, followed by introduction of universal anti-HIV screening in Western countries in 1985 and the subsequent development of direct virus detection methods for p24 antigen and viral nucleic acids of HIV-1 and HIV-2 for plasma (and blood) donors.

In the current study we have assembled a large collection of unused FVIII and FIX clotting factor concentrates with expiry dates spanning the period from 1974 to 1992. Preserved in lyophilized form, they represent a "time capsule" that provides a unique record of the range blood-borne viruses circulating in the donor population in the 1970s and 1980s and potential contributory factors to transmission risk to PWHs. These include viral loads, and effects on virus detection following introduction of virus inactivation methods and donor screening and selection policies.

2 | MATERIALS AND METHODS

2.1 | Clotting factor concentrates

Archived concentrates were purchased for therapeutic use and stored at the recommended temperature of 4°C between 1974 and around 2015. Clotting factors were subsequently transferred to a −20°C clinical laboratory freezer. Clotting factors were reconstituted immediately before testing. Expiry dates ranged from 1974 to 1992 (Table 1). Information on manufacturer and lot number was recorded; commercially manufactured clotting factors were assumed to derive from remunerated donors; those from the UK Blood Products Ltd and the French blood service were from nonremunerated donors (Table 1; column 3). FVIII and FIX ampoules were reconstituted in the indicated therapeutic volume using dH_2O.

2.2 | Nucleic acid extraction

Total nucleic acid was extracted from 1400 µL of resuspended clotting factor, using proportional volumes of buffers from the QIAamp Viral RNA kit (QIAGEN) and columns from High Pure Viral Nucleic Acid Large Volume Kit (Roche). Nucleic acid was eluted in 60 µL of RNase-free water and stored at −70°C for subsequent use.

2.3 | Real-time polymerase chain reaction (RT-PCR) screening

HCV and HIV-1 RNA sequences were detected by calibrated RT-qPCRs (700 µL test volume; Abbott Alinity M) and Micropathology Ltd. laboratories (5800, 500 µL test volume; Roche Cobas). HBV was detected and quantified using 5 µL extracted nucleic acid.[18] HEV RNA was detected by RT-qPCR assay as previously described[19] primers modified by a 5′ flap region[20] and alternative 5′-reporter and 3′-quencher dyes (MAF, TAMRA). Viral load measurements were calibrated to IU/mL using external standards from the National Institute for Biological Standards and Control.

HAV RNA as previously described[21] but without multiplexing for B19V. In-house assays for HPgV-1/-2 RNA sequences and for PARV4 and B19V DNA were used to detect and provide a relative quantitation of viral loads in the absence of external standards. Complementary DNA (cDNA) template for RNA PCR was generated by random hexamer primed synthesis using 20 µL of extracted nucleic acid to reconstitute lyophilized RNA to cDNA (EcoDry Premix; Takara Bio) without template dilution. Relative viral loads (RVLs) were calculated based on an assumption that C_t values of less than 40 (assay sensitivity limit) contained 1 or more copies in the reaction. Based on a sample representation of 23 µL in the PCR, this translates to an assay sensitivity of 43 target copies/mL). RVLs were calculated for samples with lower C_t values (ObsCt) using the formula $2^{(40\text{-ObsCt})}$.

HPgV-1, PARV4, and B19V sequences were amplified by newly designed RT-PCR assays using conserved sense and antisense primers. Assays used either specific probe hybridization or SYBR Green detection of the amplified product (Supporting Information S1: Table S1). One microliter of cDNA template was used for both RNA and DNA viruses in a 15 µL real-time reaction with 7.5 µL of 2× qPCRBIO SyGreen Blue Mix (PCRBIO) and 400 nM each primer. Reactions were run at 95°C/2 min, then 40 cycles of 95°C/5 s and 60°C/30 s with fluorescence detection, followed by a melt curve generation between 70°C and 90°C.

2.4 | HCV genotype and HIV-1 subtype analysis

RNA from samples positive for HCV RNA was amplified in the core region as previously described.[22] A new inner antisense primer Supporting Information S1: Table S1) was designed to better accommodate polymorphisms between genotypes. Amplified DNA from the 2nd round PCR was directly sequenced by Illumina (GeneWiz) next-generation sequencing (NGS) to generate approximately 100,000 paired end reads per sample. Genotype and subtype assignments were determined using a bespoke pipeline (Kraken2[23]), followed by trimming to remove adapters and low-quality reads (Trimmomatic). Reads were then mapped to a collection of 140 HCV reference sequences* (BWA-Mem2) and statistics were collected for reads aggregated to each mapped reference (Samtools). All data analysis, scripting, and plotting was conducted using Python 3.10.

HIV-1 was amplified from RT-qPCR screen-positive samples using nested primers in the p17*gag* (Supporting Information S1: Table S1) and sequenced by the Sanger method (Source BioScience).

3 | RESULTS

3.1 | Detection of HIV-1 and hepatitis viruses in clotting factors

We first investigated the degree of contamination of plasma-derived clotting factors used for hemophilia treatment between the early 1970s and into the 1990s for currently screened blood-borne viruses (HCV, HIV-1, HBV, and HEV). Samples comprised 16 commercial preparations of factor VIII or IX and eight UK- or French-origin FVIII preparations; although dates of collection of plasma used for the clotting factors were not available, we were able to record their expiry dates, providing an approximation to the time course of plasma collection perhaps displaced by 1–2 years.

Extracted RNA/DNA was assayed by standardized quantitative RT-qPCRs (Roche Cobas 5800 and Abbott Alinity) for HCV and HIV1/2 RNA sequences (Figure 1). All ($n = 12$) commercial clotting factors with expiry dates between 1976 and 1990 were HCV RNA-positive by qPCR while those with expiry dates after 1990 and which would have been universally virally inactivated by solvent/detergent

TABLE 1 Clotting factor information and testing results.

Study no	Product/manufacturer	Rem[a]	Batch no.	Expiry date	Volume	HCV[b] (IU/mL)	HIV-1 (IU/mL)	HBV (IU/ml)	HEV (IU/ml)	HPgV-1 Ct	PARV4 Ct	B19V Ct	HAV Ct
S6	Factor IX/BPL[c]	N	3C30912	01/10/1974	100	20	<10	<10	<50	40.0	40.0	40.0	40.0
S60	Factor IX/BPL trial	N	C319-6	01/01/1975	100	<10	<10	<10	<50	40.0	40.0	40.0	40.0
S5	Factor VIII/BPL	N	646	01/06/1975	100	<10	<10	<10	<50	40.0	40.0	40.0	40.0
S41	Prothromplex/ Immuno AG	R	05D474	29/11/1975	10	<10	<10	<10	<50	33.7	40.0	36.0	40.0
S42	Prothromplex/ Immuno AG	R	51274	08/02/1976	10	867	<10	<10	<50	34.1	37.0	33.3	40.0
S30	Fraction R/Immuno AG	R	05A0575	03/12/1976	10	85	<10	<10	<50	32.8	40.0	18.6	40.0
S51	Feiba/Immuno AG	R	05A0576	28/10/1977	20	1091	<10	<10	<50	33.7	40.0	35.6	40.0
S74	Factorate/Armour	R	T33403	01/05/1981	20	32,500	<10	25	<50	32.3	40.0	35.3	40.0
S72	Factorate/Armour	R	T51309	01/01/1982	20	8427	<10	<10	<50	32.8	40.0	40.0	40.0
S70	Factorate/Armour	R	B322303	01/04/1983	20	31,400	20	<10	<50	31.9	33.1	35.1	40.0
S49	Kryobulin/Armour	R	09M02381	01/04/1983	20	147,048	10911	<10	<50	29.2	40.0	22.1	30.1
S88	Factorate/Armour	R	V32303	01/04/1983	20	28,400	<10	<10	<50	31.5	32.5	35.5	40.0
S25	Factor VIII/BPL	N	HLB3084	01/07/1984	15	174	<10	<10	<50	34.6	40.0	40.0	40.0
S90	Profilate/Alpha	R	A31930	01/09/1984	20	82,800	<10	<10	<50	33.4	35.3	32.6	40.0
S66	Factorate/Armour	R	X24302H	01/09/1984	20	11,399	<10	<10	<50	31.6	40.0	40.0	40.0
S68	Factorate/Armour	R	X45507H	01/07/1985	20	17,000	42	<10	<50	28.2	40.0	36.6	40.0
S7	Octa-VI/Octapharma	R	880808-0	01/07/1989	20	<10	<10	<10	<50	40.0	40.0	30.0	40.0
S83	Factor VII/BPL	N	7D221H	27/09/1989	20	<10	<10	<10	<50	36.8	40.0	38.3	40.0
S59	Factor 8Y/BPL	N	FHC0140	21/02/1990	10	<10	<10	<10	<50	40.0	40.0	37.1	40.0
S1	Factor IX/BPL	N	FJA0060	26/09/1990	20	32	<10	<10	<50	40.0	40.0	40.0	40.0
S77	Antithrombin III/BPL	N	AT2281	04/12/1990	10	<10	<10	<10	<50	40.0	40.0	36.4	40.0
S47	VWF/Biotranfusion, France	N	87800070	01/09/1991	20	<10	<10	<10	<50	40.0	40.0	35.5	40.0
S57	Octaplas/Octapharma	R	1281496	01/07/1992	200	<10	<10	<10	<50	40.0	40.0	40.0	40.0
S58	Antithrombin, Biotranfusion	N	11000310	01/10/1992	10	<10	<10	<10	<50	40.0	40.0	40.0	40.0

[a]Remuneration status of the donors; N: Non-remunerated; R: remunerated.

[b]Positive sample shaded in yellow.

[c]BPL: Blood Products Laboratory, UK.

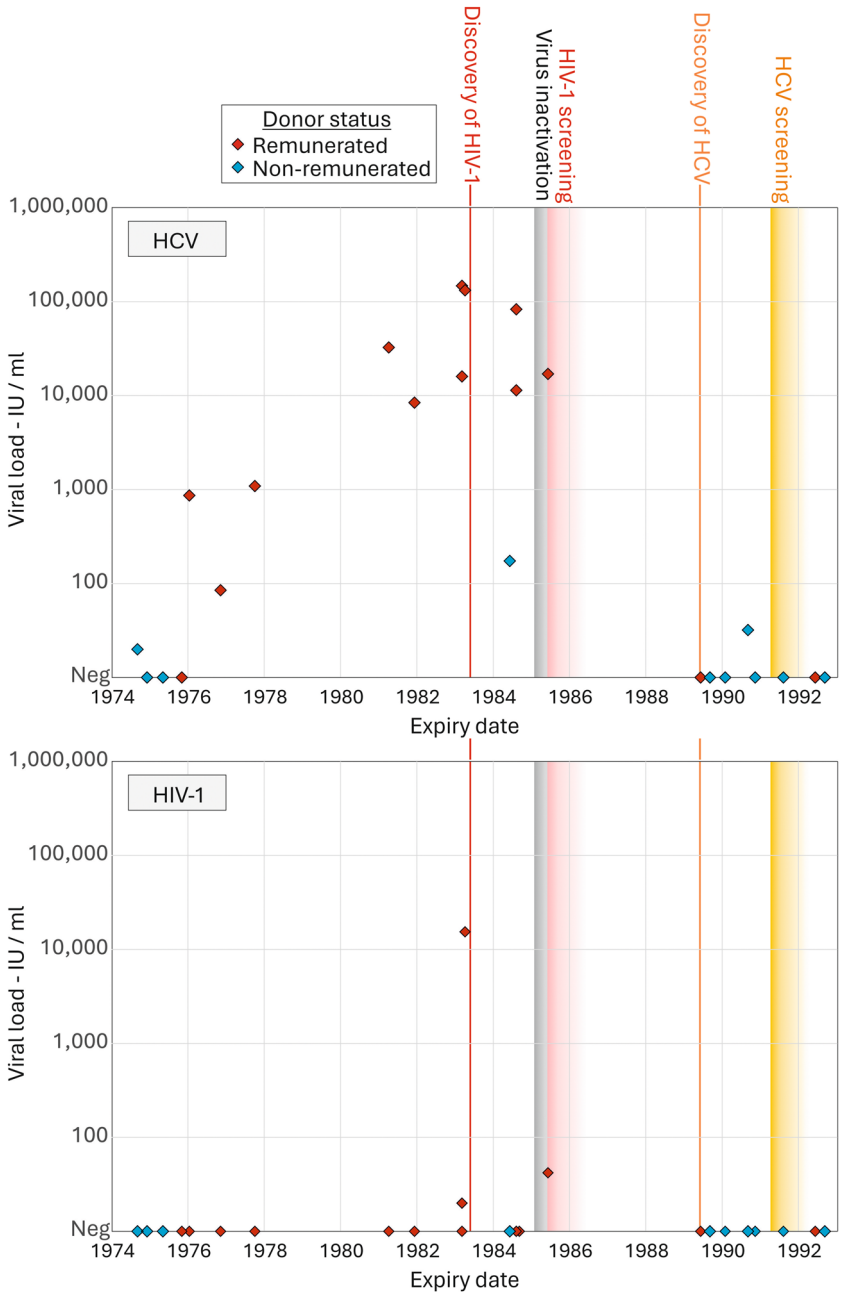

FIGURE 1 Viral loads of HCV and HIV-1 detected in clotting factors. Plot of viral loads for the commercial (red) and UK/French origin (blue) factor VIII and IX clotting factors plotted against manufacturers' expiry date (raw data provided in Table 1). The approximate timing of the introduction of measures to reduce viral contamination (HIV, HCV screening, and virus inactivation) are indicated by vertical bars). Remuneration status of the donors is indicated by red and blue symbols.

(SD)/wet heat or by dry heat were negative ($n = 4$) as was the FVIII preparation with an expiry date before 1976. Clotting factors with expiry dates 1974–1981 and likely used from the later 1970s and early 1980s showed systematically extremely high viral loads (10^4–10^5 IUs/mL) and were therefore potentially highly infectious (see Section 4). Only one clotting factor from nonremunerated donors was HCV positive (expiry date 1984), although the limited sampling possible (8/9 had expiry dates in the early 1970s or after virus inactivation from 1986) prevented a comparison of their virus contamination with that of commercial products.

HIV was much less frequently detected, with low/medium levels in three commercial clotting factors with expiry dates before 1984 (range <20–10,911 IUs/mL) and therefore collected before the discovery of HIV-1 and introduction of donor screening. All noncommercial clotting factors were negative although the limited sampling prevented a full comparison with commercial products. Of the three positive samples, only S49 with the higher viral load could be amplified using nested primers in the *gag* gene region for genetic characterization. The HIV-1 variant was of subtype B and showed no polymorphic sites, consistent with contamination from a single donor (Supporting Information S1: Table S2). The closest matched RNA-derived HIV-1 sequence on GenBank was HIV-1 strain SF20 amplified from a serum sample from a male homosexual in California, USA archived in 1978 (accession number KJ704794)[24] with 99% sequence identity.

All samples were screened using a recently described ultrasensitive real-time PCR for HBV DNA sequences.[18] One sample (S74;

Factorate, Exp. Date: May 1981, HCV-positive, HIV-1/2 negative; Appendix I) was reactive with a viral load of 20 IU/mL (and 29 and 31 IU/mL on repeat replicate testing), with all others negative (assay sensitivity [LD_{95}] of 10 IU/mL). All samples were further tested for HEV RNA by in-house RT-qPCR and were negative. HAV was detected in one commercial concentrate (Kryoglobulin; expiry date 1983).

3.2 | Detection of other blood-borne viruses

Four further viruses associated with acute or persistent viraemia on infection were screened by semi-quantitative PCR (Figure 2). HPgV-1 sequences were detected in all batches of commercial clotting factor before 1989, with batches from the earlier expiry dates also showing relatively high viral loads that contrast with the lower or undetectable

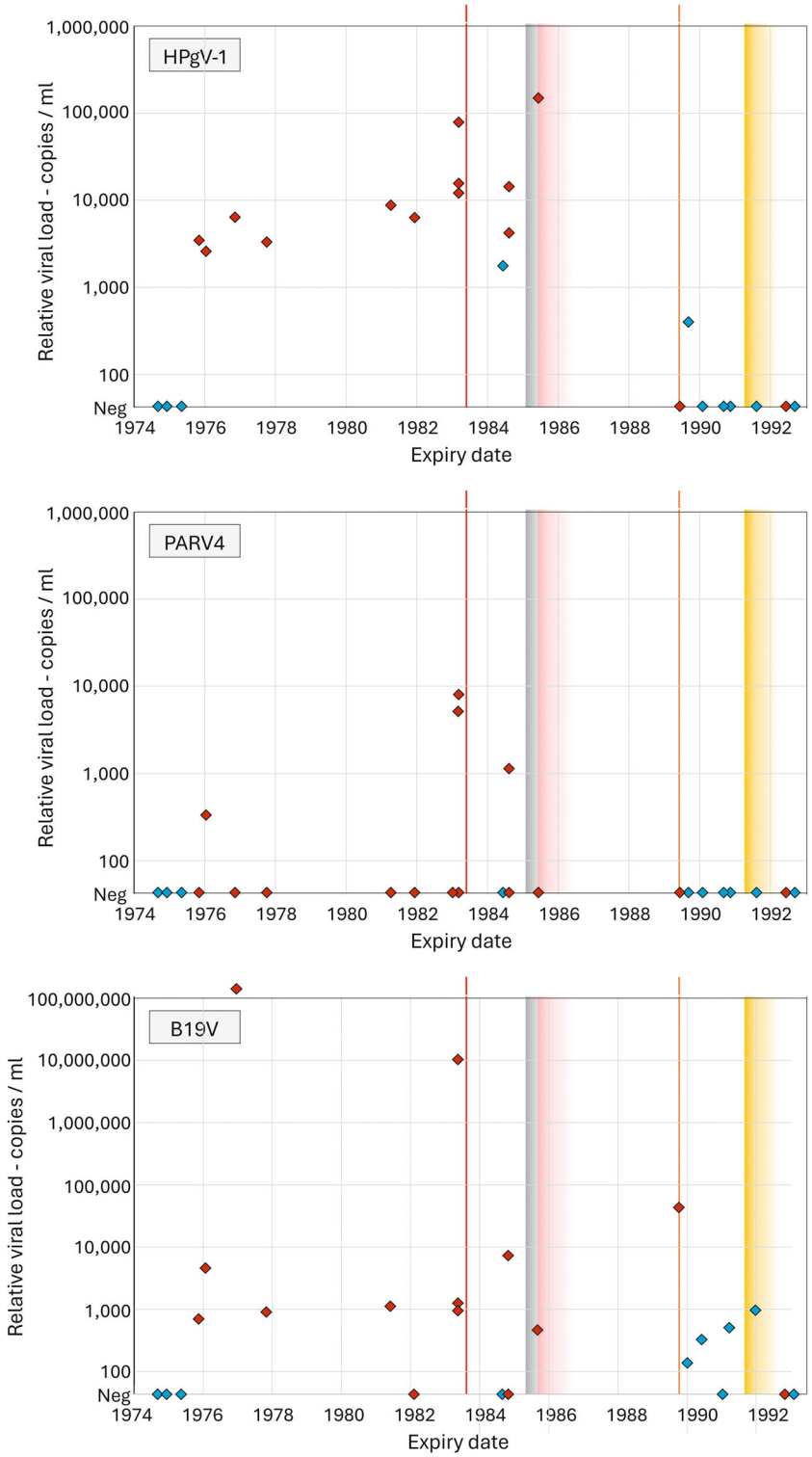

FIGURE 2 Relative viral loads of other blood-borne viruses detected in clotting factors. Plot of viral loads of HPgV-1, PARV4, and B19V in clotting factors. Symbols and reduction measures as described in Figure 1. Viral loads were calculated as describe in Methods section—each reaction had a sample representation of 23 µL, and theoretical conversion factor of 43 to express nominal viral loads to copies/mL.

HCV viral loads in these four samples. However, similarly for HCV, all samples with expiry dates after 1990 were PCR-negative, potentially the result of the introduction of virus inactivation methods leading to degradation of viral RNA. Comparable results were observed from clotting factor manufactured from non-remunerated donors. However, the samples collected in the very early 1970s from small donor pools were negative for HPgV-1, as were all samples for HPgV-2.

PARV4 was less frequently detected (4/14) in commercial clotting factors and 0/10 in those manufactured from nonremunerated plasma donors. Contrastingly, B19V was extremely frequently detected in commercially prepared clotting factors (11/14) but less frequently in UK/French products (4/10). Relative viral loads showed no temporal trend, consistent with its primarily respiratory route of transmission and consequent lack of association with risk factors for HIV-1 and HCV infection. In contrast to HPgV-1 detection, B19V was frequently detected at moderate viral loads in products with expiry dates beyond the adoption of potent virus inactivation methods (see Section 4).

3.3 | HCV genotypes detected in clotting factors

HCV core gene sequences were amplified by nested PCR from a selection of HCV-positive clotting factors with expiry dates ranging from 1976 to 1985 (Figure 3). PCR used the original core region primers,[22] and a modified assay with a more conserved inner antisense primer (see Section 2). The identification of HCV genotypes is complicated by the likelihood of multiple infected donors contaminating the same batch of clotting factor, and therefore the amplicon product was analysed through Illumina sequencing and paired-end reads. HCV reads from each PCR product which were assigned to different genotypes through comparison to a reference data set of all currently assigned HCV genotypes and subtypes using our bespoke pipeline (see Section 2; Figure 3). Distributions of HCV genotypes were comparable between the original and modified primers (Supporting Information S1: Table S3); with similar levels of genetic diversity recorded by each.

As expected, there was substantial genetic diversity of HCV strains in the six clotting factors, with genotypes 1a, 1b, 2a, 2b, and 3a represented. The most commonly observed genotype was 2b, but with frequent representation of 1a and 1b and less commonly 3a. There were no evident associations between diversity and viral loads and no clear temporal trend in genotype representation with the samples available for analysis.

4 | DISCUSSION

The study demonstrated highly variable frequencies and viral loads of a range of blood-borne viruses that contaminated plasma-derived blood products used to treat hemophilia until their replacement in the mid-1990s by recombinant FVII and FIX proteins. Findings of

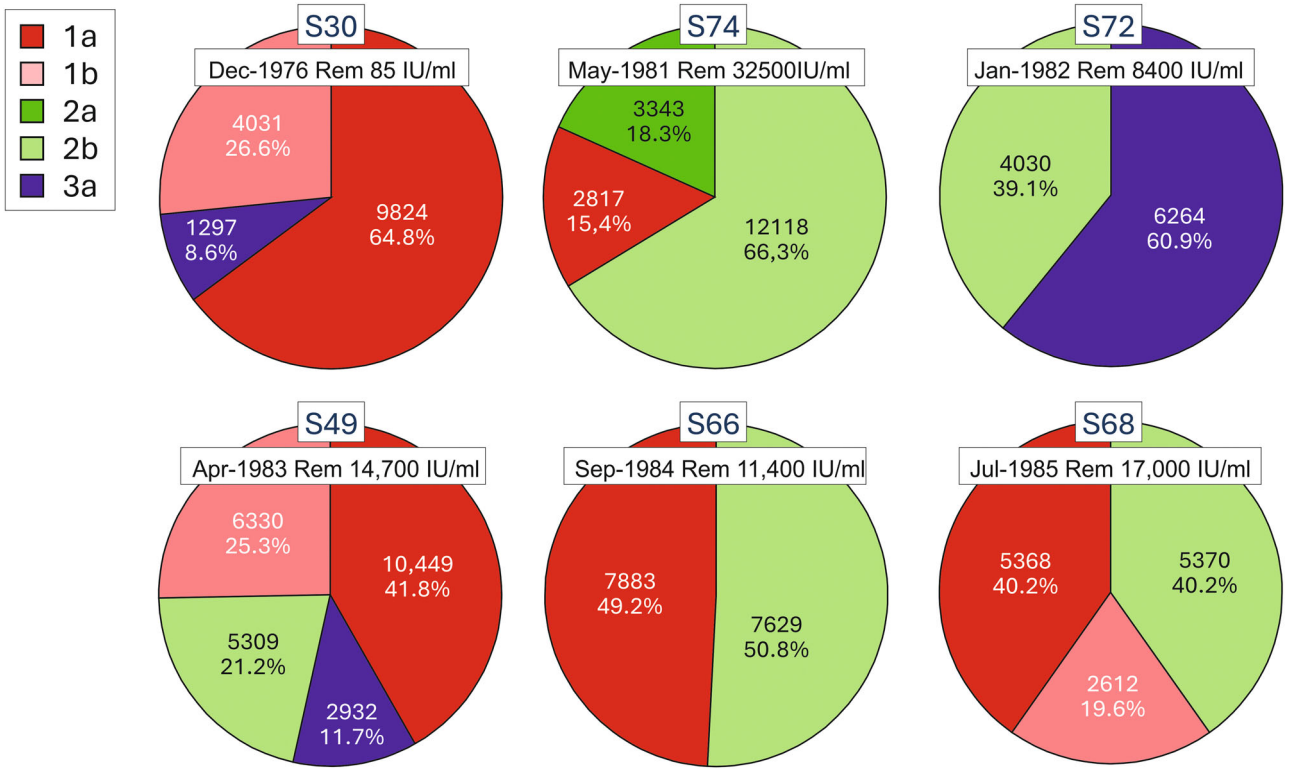

FIGURE 3 Distribution of HCV genotypes and subtypes in clotting factors. Read totals and proportions of totals of HCV reads mapped to reference sequences of currently classified genotypes and subtypes (n = 140).

frequent detection of HCV (14 from 24 clotting factors), often at viral loads (7 greater than 10,000 IU/mL) were consistent with high detection rates of HCV RNA in previous analyses of clotting factor concentrates manufactured before the introduction of virus inactivation measures in the late 1980s.[25–27] The detection of HCV RNA in clotting factors with expiry dates in the 1970s, albeit at 10–100-fold lower viral loads than those in the mid-1980s, matches clinical observations for high frequencies of NANBH in PWHs treated with pooled products.[28,29]

High viral loads were detected in clotting factors throughout the period from 1976 to 1985 in the range 10^4–10^5 IU/mL (Figure 1A). Levels were not dissimilar from viraemia levels in HCV-infected individuals and imply high frequencies of active HCV infection in donors for commercial clotting factors. This is consistent with a retrospective study that reported a 10% anti-HCV seroprevalence in paid plasmapheresis donors for a commercial manufacturer in the United States,[30] and the previously described partitioning and concentration of most HCV virions from source plasma into cryoprecipitate used to manufacture FVIII concentrate.[31] Clotting factors would likely possess an extraordinarily high infectious load for HCV, with 20–50 mL volumes repeatedly transfused to a PWH during a bleeding episode. HCV can however be readily transmitted from needlestick accidents or shared needles used for injecting drug use and tattooing with blood volumes of as little as a few microlitres. It can therefore be assumed that treated PWHs would have been repeatedly exposed to a range of HCV genotypes over the period before virus inactivation of factor VIII and IX.

The lack of detection of HCV and almost all other blood-borne viruses in clotting factors with expiry dates in the early 1970s (0/4) differed from high detection rates observed subsequently. It is possible that prolonged storage may have led to virus (and genome) degradation over time, although the desiccation from the freeze-drying process under vacuum is a highly effective means to preserve protein function and virus infectivity, and widely used in the distribution of live attenuated virus vaccines where reliable preservation of infectivity is required. A more likely factor underlying nonvirus detection is the small pool sizes (100–200 L) used to manufacture clotting factors at that time (Dr. R. Perry; personal communication). Such volumes would derive from a few hundred nonremunerated plasma donors, lower than the viraemia frequencies for most or all viruses screened for in the study.

We used Illumina NGS to quantify the relative frequencies of different HCV genotypes in the clotting factors used in the 1970s and 1980s in amplicons from the conserved core genome region. This confirmed the presence of a variety of HCV genotypes in each product, representing a multiplicity of infected donors contributing to each batch of clotting factor analysed. These findings are consistent with the same wide diversity of HCV genotypes infecting PWHs in England and Scotland, with genotypes 1a, 1b, 2a, 2b, and 3a almost exclusively recorded,[32,33] and a high frequency of mixed infections and frequent changes in genotype in longitudinal studies consistent with multiple infection episodes.[33,34] We have previously shown that genotype frequencies in PWHs closely matched those in the donor populations from which the clotting factors were manufactured.[35] The finding of high frequencies of genotypes 1a and 1b in commercial clotting factors manufactured from primarily US plasmapheresis donors (Figure 3) is therefore consistent with high frequencies of detection of these genotypes in PWHs treated with commercial product. However, the high detection frequencies of genotypes 2a and 2b (five from six samples analysed) was unexpected and may reflect an earlier pattern of HCV genotype circulation in the US and elsewhere in the decades before the discovery of HCV.

Sporadic detection of HIV-1 in clotting factors[36] was similarly consistent with the infrequent detection and low viral loads of HIV-1 in clotting factors with expiry dates between 1983 and 1985 in the current study (3/24) and likely use in the 1–2 preceding years. These correspond to the period between 1979 and 1985 in which the majority of HIV-1 seroconversions of PWHs in the United Kingdom treated with imported FVIII or FIX occurred.[37] We found a close genetic relationship between the HIV-1 strain from a commercial cryoprecipitate prep (S49; exp. date in 1984) with the SF20 strain recovered from a very early archived serum sample from a male homosexual in California in 1978.[24] This is a member of the very early lineages that initially circulated in San Francisco before the subsequent AIDS pandemic and consistent with suspected origins of HIV-1 infecting PWHs around that time.

The degree of virus contamination of plasma-derived products is potentially influenced by several distinct variables. These include the frequency of infection in the donor population and the duration and level of viraemia following infection. The ability of HCV to establish persistent infection in the majority of those infected with ongoing high-level viraemia is thus likely to represent the primary factor in the extremely high rates of detection in the clotting factors. Widespread distribution and a long-term persistence rate of around 20%–25% in those infected with HPgV-1 as adults is similarly consistent with its high rate of detection in this (14/24) and previous studies.[38,39] As for HCV, HPgV-1 viral loads in clotting factors in the period 1974–1985 of between 1000 and 100,000 nominal copies/mL were not dissimilar from the geometric mean of 3,000,000 RNA copies/mL in the high viral load cluster in a previous investigation of blood donor viral loads.[14] These findings contrast with the complete absence of clotting factors positive for the closely related HPgV-2 but are however consistent with likely extreme rarity of HPgV-2 infections even in HCV-infected people who inject drugs (PWIDs) and apparent infrequent persistence of infections.[40,41]

Detection frequencies were however also relatively high for viruses such as B19V and PARV4 (15/24 and 4/24, respectively) associated with acute resolving infections and relatively short durations of viraemia. In the case of B19V, the relatively high population incidence (with 30%–50% of donors typically with serological evidence for past infection) combined with extremely high acute viraemia levels and the large pool sizes used to make clotting factors in the 1980s undoubtedly contributed to the near universal contamination of clotting factors throughout the study period.

Viraemia levels in acute PARV4 infections are less clearly delineated, but its almost exclusive association with PWIDs in Western countries and low incidence of infection in the general population may have contributed to its less frequent detection (5/18 pre-1986, consistent with[42,43]) and lower viral loads compared to B19V. While B19V continued to be detected after the introduction of virus inactivation methods from the mid-1980s, consistent with the known stability of parvoviruses to solvent-detergent and heart inactivation methods,[44] the disappearance of PARV4 in clotting factors with expiry dates after 1986 suggests that other measures, such as screening for HIV-1 and implementation of enhanced donor selection to exclude those with known risk factors for blood-borne virus infections, may have contributed to the observed reduction in PARV4 (and HCV).

The infrequent detection of HBV DNA in the study samples (1 from 24) using a highly sensitive PCR[18] matches previously published findings of uniform negativity of clotting factors with expiry dates before 1990 in a study using a likely less sensitive PCR assay.[45] It was commented at the time that these negative results were inconsistent with remarkably high frequencies of past exposure to HBV in PWHs, with antihepatitis B core antibodies (anti-HBc) reported in >80% receiving nonvirally inactivated concentrate,[45,46] substantially higher than the background population anti-HBc seroprevalence in Western countries of typically <3%. However, frequent transmission of HBV from clotting factors manufactured from HBsAg-negative-screened plasma has been described,[46,47] potentially the outcome of including donors with occult HBV infections with undetectable HBsAg and low levels of infectious HBV particles.[48] Detection frequencies of other human hepatitis viruses, HAV and HEV were low, with only one batch of cryoglobulin from 1983 positive by PCR for HAV RNA. The absence of detectable HEV is consistent with the absence of clear evidence of higher rate of past exposure to PWHs receiving nonvirally inactivated clotting factors in retrospective sero-epidemiological surveys.[49,50] HAV transmission been reported from several centers associated with the use of solvent detergent inactivated factor VIII between 1989 and 1992 (reviewed in Vermylen & Peerlinck[51]), but without unequivocal evidence for increased seroprevalence in PWHs.[52,53]

As an analysis of the relationship between clotting factor contamination and infection of PWHs treated in the 1970s–1980s, the study has limitations, including a lack of numerical power to analyse the separate contributions of a large number of possible variables influencing blood product infectivity, including pool size used for clotting factor manufacture, virus epidemiology in the donor population, degree of persistence in donors and PWHs, resistance to virus inactivation and donor selection. Second, while expiry dates of the individual clotting factors were recorded, these do not have a fixed temporal relationship with manufacture or donation time, preventing precise matching of factor VIII/IX contamination with infection of PWHs. Nevertheless, the study does record the extraordinary diversity and frequent high viral loads of a wide range of blood-borne viruses that PWHs were exposed to from their therapy over a prolonged period.

The combined analyses of epidemiologically and physically distinct viruses provides a valuable framework to compare effects of interventions, such as virus inactivation and more effective donor screening on viral loads and likely infectivity. Future investigations will use agnostic metagenomic NGS methods to expand the analysis of the range and genetic diversity of viruses in the clotting factors and further and further characterize virus exposure in this patient group who have been historically sadly affected by this issue.

AUTHOR CONTRIBUTIONS

Michael Makris, **C. Patrick McClure**, **Peter Simmonds**, **Alexander W. Tarr**, **William L. Irving**, **Tanya Golubchik**, **Heli Harvala**, **Judy Breuer**: Study design, conceptualization. **C. Patrick McClure**, **Kai Kean**, **Kaitlin Reid**, **Michael X. Fu**, **Piya Rajendra**, **Shannah Gates**: Laboratory testing. **Peter Simmonds**, **C. Patrick McClure**, **Heli Harvala**: Manuscript preparation. **C. Patrick McClure**, **Kai Kean**, **Kaitlin Reid**, **Richard Mayne**, **Michael X. Fu**, **Piya Rajendra**, **Shannah Gates**, **Judy Breuer**, **Heli Harvala**, **Tanya Golubchik**, **Alexander W. Tarr**, **William L. Irving**, **Michael Makris**, **Peter Simmonds**: Manuscript review, editing.

ACKNOWLEDGMENTS

This article was funded by the National Institutes for Health Research (NIHR) grant code NIHR203338.

CONFLICT OF INTEREST STATEMENT

Michael Makris has received honoraria for lecturing, grant reviewing, and advisory committee participation from NovoNordisk, Takeda, Grifols, and Sanofi. The other authors declare no conflict of interest with publishing this manuscript.

DATA AVAILABILITY STATEMENT

All original data obtained in the study is available from the authors on request.

ETHICS STATEMENT

All testing was performed on commercially sourced clotting factor concentrates; the study did not involve testing of any human samples and no patient data were used. Therefore, no ethics approval was required.

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ENDNOTE

* https://ictv.global/sg_wiki/flaviviridae/hepacivirus/Table1

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SUPPORTING INFORMATION

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

How to cite this article: McClure CP, Kean K, Reid K, et al. Reconstruction of the historic time course of blood-borne virus contamination of clotting factor concentrates, 1974–1992. *J Med Virol*. 2024;96:e29774. doi:10.1002/jmv.29774