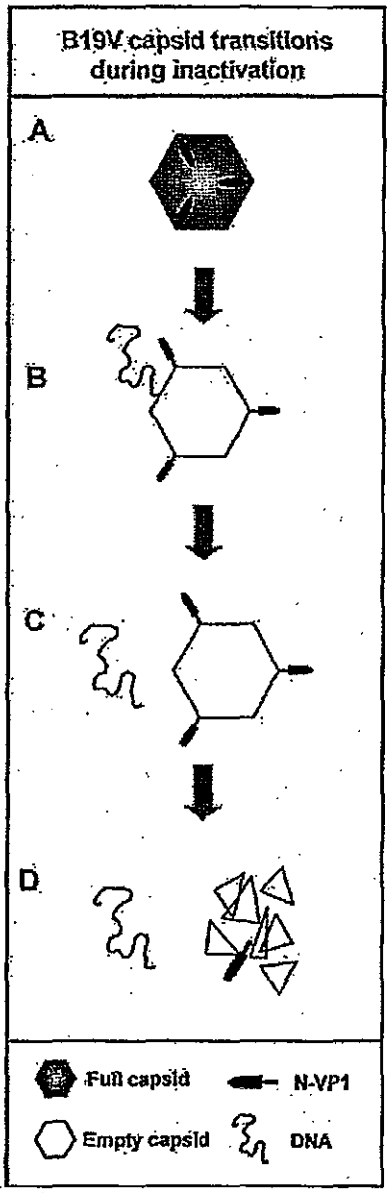

Fig. 6. Schematic representation of the B19V capsid structural transitions during inactivation. The first structural rearrangements observed after mild heat or low-pH treatments of B19V is the externalization of N-VP1 sequences,[36] including the PLA_2 motif and the accessibility of the viral DNA.[28] At higher temperatures or more acidic conditions, the viral DNA is dissociated from the capsid. Finally, the viral particle is disintegrated.

heat or low pH.[28] Therefore, although not infectious, the inactivated capsids are enzymatically active. The binding of the PLA_2-active capsids to cells (Fig. 4), whether specific or not, might still have certain biologic effect. It seems very unlikely, however, that such effects could be elicited through the administration of plasma-derived products containing inactivated B19V intact capsids. First, there may not be any intact capsids present in plasma-derived products due to the application of procedures of virus removal and/or inactivation, which are by far stronger than the ones applied in the present studies. Second, to elicit biologic activities other than virus replication, a large amount of B19V capsids or genomic viral DNA would be required. Synoviocyte migration for instance has been shown only to occur at a concentration of 10^{11} virions per mL.[41] Also, Norbeck and colleagues[38] use 10^{12} protein molecules per mL in an assay that showed the inhibition of hematopoiesis by VP2. Such high concentrations are simply not possible in plasma-derived products.

In summary, the molecular mechanism underlying the inactivation of B19V has been elucidated. The first structural transition determining B19V inactivation is not the disintegration of the capsid but the release of the viral DNA. Comparison studies revealed that although the DNA release from intact capsids seems to be a common feature within the *Parvoviridae* family, it occurs much more promptly and to a higher extent in B19V, explaining its lower resistance to inactivation treatments.

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				2007 年 11 月 29 日	該当なし	

一般的名称	人ハプトグロビン	研究報告の公表状況	Vox Sanguinis 2007; 93: 341-347	公表国 イギリス
販売名（企業名）	ハプトグロビン注ーヨシトミ(ベネシス)			

研究報告の概要

<背景及び目的>パルボウイルスは小さな非エンベロープの DNA ウイルスで、ウイルス不活化処理に対して比較的抵抗性がある。最近確認されたヒトパルボウイルス PARV4 が、類縁のジェノタイプ 2 型ウイルス（PARV5）を含め、血漿分画製剤の製造に使用されたプール血漿に混入していることが分かった。本報告では PARV4 が凝固因子製剤中に存在するのか否かを決定するための調査について述べる。
<材料及び方法>過去 30～35 年間に製造された第Ⅷ因子製剤について PARV4 及び B19 シーケンスのスクリーニングを実施した。PARV4 陽性製剤中の PARV4 ウイルス量は TaqMAN 分析法で測定し、DNA シーケンス分析によりジェノタイプを確認した。
<結果>第Ⅷ因子製剤 175 ロットのうち 28 ロットが PARV4 シークエンスを含み、その内 2 ロットにジェノタイプ 1 型及び 2 型の両方が存在することが分かった。最大ウイルス量は 10^5copies/mL 以上であった。PARV4 陽性の第Ⅷ因子製剤の大部分は 1970 年代及び 1980 年代に製造されていた。B19 もまたこれらの製剤をしばしば汚染していた。
調査した 175 ロットの PARV4 DNA 陽性ロットを有効期間別に区分すると、有効期間が 1974-1989 年のロットでは 23%（27/115）が陽性であったのに対して、1990-2005 年では 2%（1/60）が陽性であった。
<結論>PARV4 は第Ⅷ因子製剤の 16%、特に 1970 年代及び 1980 年代の古いロットから検出された。これらの製剤からのウイルス安全性及びレシピエントへの感染可能性の重要性は、依然不明である。

報告企業の意見	今後の対応
過去 30～35 年間に製造された第Ⅷ因子製剤から PARV4 シークエンスが検出されたとの報告である。 PARV4 が発見されたのは 2005 年であり、PARV4 及びその関連変異型である PARV5 の病原性は現時点では明らかではない。今後も注意深く PRV4 に関する追加情報をフォローする必要があると考える。	PRV4 に関連する情報については、今後も注視することとする。

使用上の注意記載状況・その他参考事項等

1. 慎重投与（次の患者には慎重に投与すること）
(4) 溶血性・失血性貧血の患者〔ヒトパルボウイルスＢ19 の感染を起こす可能性を否定できない。感染した場合には，発熱と急激な貧血を伴う重篤な全身症状を起こすことがある。〕
(5) 免疫不全患者・免疫抑制状態の患者〔ヒトパルボウイルスＢ19 の感染を起こす可能性を否定できない。感染した場合には，持続性の貧血を起こすことがある。〕
2. 重要な基本的注意
(1) 略
1) 血漿分画製剤の現在の製造工程では、ヒトパルボウイルスＢ19 等のウイルスを完全に不活化・除去することが困難であるため、本剤の投与によりその感染の可能性を否定できないので、投与後の経過を十分に観察すること。
5. 妊婦，産婦，授乳婦等への投与妊婦又は妊娠している可能性のある婦人には、治療上の有益性が危険性を上回ると判断される場合にのみ投与すること。〔妊娠中の投与に関する安全性は確立していない。本剤の投与によりヒトパルボウイルスＢ19 の感染の可能性を否定できない。感染した場合には胎児への障害（流産，胎児水腫，胎児死亡）が起こる可能性がある。〕

(7)

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Human parvovirus PARV4 in clotting factor VIII concentrates

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Background and Objectives Parvoviruses are small non-enveloped DNA viruses, relatively resistant to virus inactivation procedures. The recently identified human parvovirus PARV4, including a related genotype 2 virus (also termed PARV5), has been found to be a contaminant of pooled plasma used in the manufacture of plasma-derived products. This report describes an investigation to determine whether PARV4 is present in clotting factor concentrates.

Materials and Methods Factor VIII concentrates manufactured in the past 30–35 years were screened for PARV4 and human parvovirus B19 (B19V) sequences. Viral loads in products testing positive for PARV4 were quantified using a consensus TaqMan assay designed to a highly conserved region. DNA sequence analysis was performed to confirm the genotypes present.

Results From a total of 175 lots of factor VIII concentrate, 28 of these contained PARV4 sequences, and in two lots both genotypes 1 and 2 were found to be present. The highest viral loads observed exceeded 10^5 copies per ml. The majority of factor VIII concentrates testing positive for PARV4 were manufactured in the 1970s and 1980s. Human B19V was also a frequent contaminant of these products.

Conclusions PARV4 was detected in 16% of factor VIII concentrates, particularly in older batches from the 1970s and 1980s. The significance in terms of the viral safety and potential transmission to recipients of these products is not yet known.

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Key words: factor VIII, genotype, PARV4, PARV5, parvovirus, virus contamination.

Introduction

PARV4 was originally identified in plasma from a patient with symptoms of acute virus infection following high-risk behaviour for human immunodeficiency virus 1 (HIV-1) transmission, but subsequently confirmed to be HIV-1 negative [1]. This patient was an intravenous drug user, infected with hepatitis B virus (HBV), with a range of symptoms including fatigue, vomiting and diarrhoea, sore throat, neck stiffness and joint pains. Phylogenetic analysis showed that PARV4 did not closely resemble other known human or animal parvoviruses [1].

Parvovirus B19 (B19V) is the prototype human parvovirus, infecting erythroid progenitor cells leading to erythema infectiosum, aplastic crisis, arthropathy and hydrops fetalis [2]. B19V is normally transmitted via the respiratory route; however, transmission also occurs through the administration of contaminated blood products and solvent/detergent-treated plasma and can result in clinically apparent infection [3–6]. Since 2004, European regulations have required that manufacturers of certain plasma derivatives, including anti-D immunoglobulin and plasma pooled and treated for virus inactivation, screen pooled plasma for B19V by nucleic acid amplification techniques (NAT), and this has led to a reduction in the levels of B19V present in manufacturing start pools [7]. NAT screening for B19V has now been widely implemented by manufacturers.

We have recently demonstrated the presence of PARV4 and a related variant virus (termed PARV5), in pooled plasma used in the manufacture of plasma-derived medicinal products [8]. These viruses are frequently detected in 4–5% of these pools with viral loads of up to 10^6 copies per ml of plasma.

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In the case of blood donors, PARV4 and PARV5 have been found in approximately 2% of individuals and at a higher frequency in febrile patients [9]. Sequence analysis shows that PARV4 and PARV5 share ~92% nucleotide identity over a 4860-bp region [10], similar to the level observed between B19V genotypes 1–3 [11], to which PARV4 shares ~45% nucleotide identity. At the amino acid level, PARV4 and PARV5 sequences are more conserved, and this is especially the case for the second open reading frame (ORF2), encoding the viral capsid-like protein, such that PARV4 and PARV5 are likely to represent a single serotype [10]. This sequence analysis has led to the proposal that PARV4 and PARV5 should be referred to by a single virus name, PARV4, comprising genotypes 1 and 2 (previously PARV5). In this study, we have investigated the presence of PARV4 genotypes 1 and 2 in clotting factor VIII concentrates, manufactured over the past 30–35 years. We have also examined these products for the presence of B19V.

Materials and methods

Factor VIII concentrates

Coagulation factor VIII concentrate products received at the National Institute for Biological Standards and Control (NIBSC) were stored at 4 to −20 °C until analysis. A total of 175 lots of 12 factor VIII concentrate products, from 10 manufacturers (named A–J), were investigated. Products were manufactured over a 30- to 35-year period, with expiry dates ranging between 1974 and 2005. Factor VIII product details are further described in Table 1.

Nucleic acid extraction

Factor VIII concentrates were reconstituted in sterile distilled water according to the manufacturer's instructions. Total nucleic acid was extracted from 1 ml of reconstituted concentrate using the MagNA Pure LC instrument (Roche Applied Science, Mannheim, Germany) and was eluted in 50 μl as previously described [7].

Screening for PARV4 in factor VIII concentrates

Factor VIII concentrates were initially screened for the presence of PARV4 genotype 1 and 2 sequences using a gel-based polymerase chain reaction (PCR), using primers specific to ORF2 of PARV4 [9]. We have previously confirmed the specificity and sensitivity of these primers to be one to 10 copies of PARV4 sequences. The presence of PARV4 in factor VIII concentrates was confirmed by DNA sequence analysis of amplification products. Amplicons were purified using the QIAEX Gel Extraction kit (Qiagen, Hilden, Germany). Sequencing was performed using the BigDye Terminator v1·1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), using the T7 promoter primer and the pUC/M13 reverse primer. Following removal of dye terminators, using the DyeEx 2·0 Spin Kit (Qiagen), sequencing reactions were run on an ABI 3130XL Genetic Analyser (Applied Biosystems).

Quantification of PARV4 in factor VIII concentrates

Following the initial screening of factor VIII concentrates for PARV4, viral loads in samples testing positive for these

Table 1 Detection of PARV4 and B19V in factor VIII concentrates

Product/ manufacturer	Expiry date	Number of lots tested	Purification process	Virus inactivation	Number of positive lots by PCR	
					PARV4	B19V
1/A	1974–1978	37	Precipitation	None	3	23
2/B	1976–1977	2	Precipitation	None	1	2
3/C	1976–1978	5	Precipitation	None	3	5
4/D	1977–1978	2	Precipitation	None	1	2
5/E	1977–1980	55	Precipitation	None	14	9
6/C	1985	1	Precipitation	Dry heat (68 °C, 72 h)	1	1
4/F	1985	1	Precipitation and adsorption	Wet heat (heptane) (60 °C, 20 h)	1	1
7/E	1985–1987	8	Precipitation and adsorption	Dry heat (68 °C, 72 h)	0	5
8/A	1986	4	Precipitation (plus further purification)	Steam treatment (60 °C, 10 h)	3	4
9/EGH	1997–2004	16	Monoclonal antibody	Pasteurization (60 °C, 10 h)	0	2
10/I	1998–2002	13	Monoclonal antibody	Solvent/detergent	0	7
11/I	1999–2003	13	Precipitation	Dry heat (80 °C, 72 h)	1	7
12/J	2001–2005	18	Affinity chromatography	Solvent/detergent, dry heat (80 °C, 72 h)	0	2
				Total number of positive lots/number of lots tested	28/175	70/175

viruses were determined using a real-time PCR assay designed to a highly conserved region of PARV4 as previously described [9,10]. The primers used in this assay are directed towards a region of ORF2 of PARV4 that is highly conserved between the two genotypes. A standard curve was generated from plasmid DNA containing the 103-bp ORF2 PCR product.

Detection of B19V DNA in factor VIII concentrates

Coagulation factor concentrates were additionally tested for the levels of B19V DNA using an in-house PCR assay as previously described [7]. This assay detects B19V genotypes 1–3.

DNA sequence analysis of a variable region of ORF1 of PARV4

Using a multiple sequence alignment of near full-length PARV4 genomes (GenBank accession no. DQ873386-91) [10], primers were designed to a variable region of the PARV4 genome. Primers PARV35F (5′ TTCCTACTGGATTTCTCTCCAACC 3′) and PARV596R (5′ GGTAAGGCAATAGCACCTTGAGG 3′) were used to amplify a 562-bp region of ORF1 of PARV4 (corresponding to nucleotides 317–878 of PARV4 genotype 1, GenBank accession no. AY622943, and nucleotides 151–712 of PARV4 genotype 2, GenBank accession no. DQ873390), from extracted factor VIII samples. Amplification reactions were performed using the proof-reading enzyme Phusion™ Hot Start DNA Polymerase (Finnzymes OY, Espoo, Finland) as described previously [8]. For thermal cycling, a T3 thermal cycler (Biometra, Göttingen, Germany) was used with the following cycling conditions: 98 °C for 30 seconds, followed by 45 cycles of 98 °C for 10 seconds, 59 °C for 30 seconds and 72 °C for 20 seconds. Amplicons were analysed by agarose gel electrophoresis and compared with known size markers. Amplification products were purified as before, and cloned into the pT7 Blue vector according to the manufacturer's instructions (Novagen, Darmstadt, Germany). Sequencing was performed as previously described and was analysed using the GCG software package, version 10·2 (University of Wisconsin, Madison, WI, USA). Sequences were aligned using Clustal W [12], and a neighbour-joining tree (nucleotide distance with Jukes–Cantor correction, pairwise gap deletion) with bootstrap resampling (100 replicates), was constructed using MEGA3 software [13].

Results

Contamination of factor VIII concentrates with human parvoviruses

A total of 175 lots of 12 factor VIII concentrate products, from 10 manufacturers, were examined for the presence of PARV4 and B19V DNA by PCR. The expiry dates on these lots ranged from 1974 to 2005. As shown in Table 1, 16% (28/175) of lots tested positive for PARV4, while 40% (70/175) of lots tested positive for B19V DNA. The majority of factor VIII products testing positive for PARV4 DNA had an expiry date of pre-1990 [23% (27/115) of lots expiring 1974–1989 tested positive for PARV4, while only 2% (1/60) of lots expiring 1990–2005 tested positive for PARV4 DNA] (Fig. 1). In contrast, there was no significant difference in the prevalence of B19V in factor VIII products expiring pre- and post-1990 [45% (52/115) of lots expiring 1974–1989 tested positive for B19V, while 30% (18/60) of lots expiring 1990–2005 tested positive for B19V DNA] (Fig. 1).

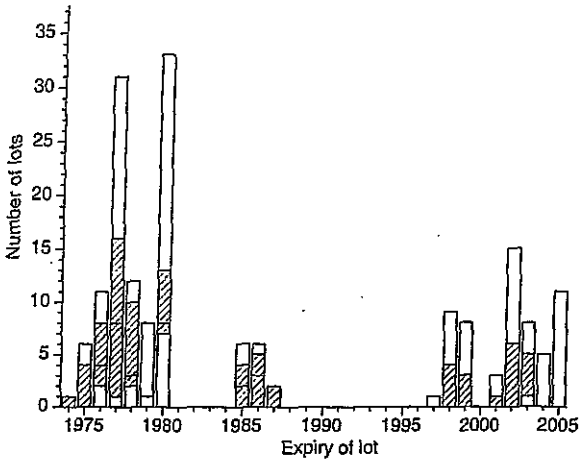

Fig. 1 Prevalence of parvoviruses PARV4 and B19V in factor VIII concentrates manufactured over the past 30–35 years. The number of lots testing positive for PARV4 (□), B19V (▨), both PARV4 and B19V (▨), and those testing negative for these viruses (□) are shown.

PARV4 ORF2 PCR products amplified by the gel-based assay were sequenced, and the majority determined to be of PARV4 genotype 2 (Table 2). In two factor VIII products both PARV4 genotype 1 and 2 sequences were amplified and sequenced. Viral loads of PARV4 in factor VIII products were determined by a consensus sequence real-time PCR assay [9], designed to detect a highly conserved region of ORF2 of PARV4. Viral loads ranged from < 100 to more than 3×10^5 copies per ml of product (Table 2), with the majority of contaminated lots containing 4–5 $\log_{10}$ PARV4 copies per ml of product (Fig. 2). The levels of B19V were as high as $2{\cdot}5 \times 10^8$ IU/ml of product (Table 2).

Manufacturing plasma pools relating to these factor VIII products were only available for the most recent factor VIII products. Factor VIII product number 28 (Table 2) had an expiry date of 2003, and was manufactured from two plasma pools 28A and 28B. Plasma pool 28A tested positive for PARV4 genotype 1 DNA by PCR with a viral load of $3{\cdot}3 \times 10^5$ copies per ml of plasma, while pool 28B tested negative for both PARV4 genotypes.

Table 2 Levels of PARV4 and B19V in factor VIII concentrates testing positive for PARV4 DNA

Factor VIII	Product/ manufacturer	Expiry date	PARV4 genotype[a]	PARV4 viral load (log_{10} genome copies per ml product)	B19V viral load (log_{10} IU/ml product)
1	1/A	1976	2	< 2·00[c]	8·40
2		1977	1	1·89	6·71
3		1977	1 & 2[b]	1·71	7·64
4	2/B	1977	2	3·11	2·59
5	3/C	1976	2	1·82	4·91
6		1977	2	3·28	5·33
7		1978	1	1·86	2·75
8	4/D	1977	2	2·48	2·22
9	5/E	1977	2	1·75	–
10		1977	2	4·10	2·39
11		1977	2	4·82	6·05
12		1978	2	4·15	–
13		1978	2	4·36	–
14		1979	2	2·66	–
15		1980	1	4·31	6·44
16		1980	1 & 2	3·01	–
17		1980	2	4·39	–
18		1980	2	5·49	–
19		1980	2	5·03	–
20		1980	2	2·37	–
21		1980	2	4·30	–
22		1980	2	2·00	–
23	4/F	1985	1	< 2·00[c]	4·57
24	6/C	1985	1	1·32	5·79
25	8/A	1986	1	4·08	7·15
26		1986	2	3·81	5·85
27		1986	2	4·53	4·36
28	11/I	2003	1	2·32	–

[a]Determined by sequencing of ORF2 amplification products.

[b]ORF2 amplification products were determined to be PARV4 genotype 1 sequences, while the amplified variable ORF1 region was determined to be PARV4 genotype 2.

[c]Factor VIII lot tested positive for PARV4 DNA by qualitative PCR but the viral load was below the level of quantification by real-time PCR, and was therefore given an arbitrary viral load of < 2 log_{10} genome copies per ml product.

–, product tested negative for B19V DNA.

Analysis of PARV4 sequences

Previous analysis of PARV4 sequences showed that ORF1 was slightly less conserved than ORF2 [10]. We therefore amplified and sequenced a 562-bp variable region at the 5′ end of ORF1 from 26/28 factor VIII concentrates testing positive for PARV4 sequences. It had not been possible to amplify the 562-bp variable ORF1 region of PARV4 from factor VIII products 7 and 9 (Table 2). Both PARV4 genotype 1 and 2 sequences were amplified from factor VIII product number 16 (Table 2). Phylogenetic analysis of these PARV4 sequences shows that they fall into two distinct genetic clusters, representing genotypes 1 and 2 (Fig. 3). Across the two genotypes, PARV4 nucleotide sequences amplified from factor VIII products differ from each other by greater than 11% over the region sequenced. Within each genotype, all PARV4 sequences amplified from factor VIII concentrates were greater than 99% homologous (at the nucleotide level, over the 515-bp region sequenced), despite products being manufactured over a 30- to 35-year period. In fact, several PARV4 genotype 1 and 2 sequences amplified from factor VIII products manufactured as early as the mid-1970s were 100% identical at the nucleotide level, over the 515-bp region sequenced, to the recently identified respective strains BR10749 (genotype 1) and BR10627 (genotype 2) [10].

Discussion

We recently demonstrated the presence of the newly identified human parvovirus PARV4 including the related genotype 2

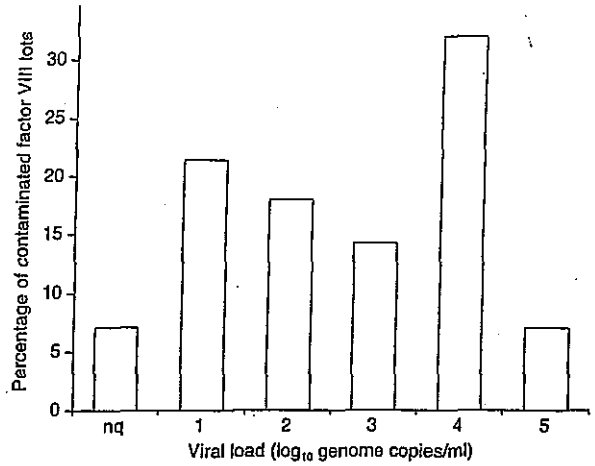

Fig. 2 Viral DNA loads of PARV4 (log_{10} genome copies per ml) in contaminated factor VIII concentrates. nq, not quantifiable.

virus (previously termed PARV5) in manufacturing plasma pools, with these viruses detected in approximately 5% of pools [8,9]. In this present study, we have detected PARV4 viruses in products derived from such plasma pools, specifically in coagulation factor VIII products, manufactured over the past 30–35 years. Information regarding the source of plasma used in the manufacture of products examined in this study was difficult to obtain as it is not provided with the products. These details could only be obtained for the most recent factor VIII product testing positive for PARV4 DNA. This factor VIII concentrate had an expiry date of October 2003, and was manufactured from two plasma pools in September 2000. Donations relating to these plasma pools were collected in or after July 1998 from paid donors from the USA. This suggests that viruses detected in these factor VIII products may date from up to 5 years prior to the expiry date on the product. Details from other manufacturers of recent factor VIII concentrates (testing negative for PARV4) also indicate that donations relating to these products were sourced up to 5 years prior to the expiry date.

The prevalence of PARV4 in factor VIII concentrates was found to be greater in products expiring pre-1990 than in those with an expiry date of post-1990. This difference in the prevalence of PARV4 in factor VIII products over time may reflect the introduction of blood safety measures from the mid-1980s in response to the HIV epidemic, in particular, the introduction of screening tests for HIV and hepatitis C virus (HCV) (in 1986 and 1991, respectively), and virus inactivation of manufacturing plasma pools (introduced in the mid-1980s). The screening of blood donations for HIV and HCV identified 'high-risk' donor groups, such as homosexual males and individuals with a history of intravenous drug use (IVDU), and these groups were subsequently excluded from donating blood [14]. Factor VIII products tested in this study

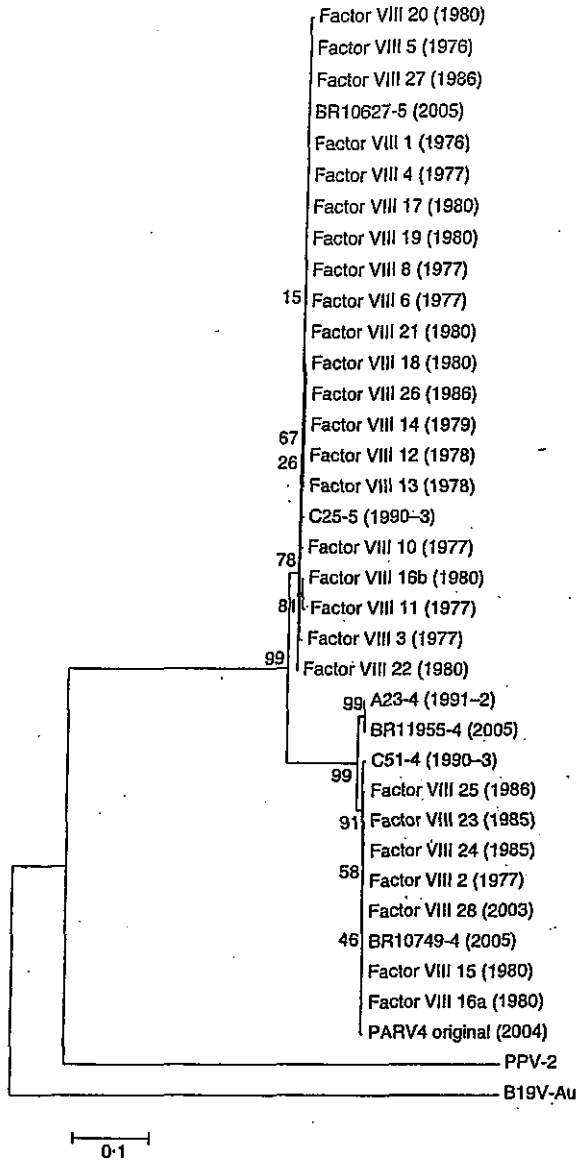

Fig. 3 Phylogenetic analysis of a 515-bp region of ORF1 of PARV4 amplified from factor VIII concentrates. Sequences are named according to factor VIII number and expiry of lot (Table 2). The alignment includes other recently sequenced strains of PARV4 genotype 1; PARV4 original (GenBank accession no. AY622943), BR10749-4 (GenBank accession no. DQ873386), BR11955-4 (GenBank accession no. DQ873388), A23-4 (GenBank accession no. DQ873389) and C51-4 (GenBank accession no. DQ873387); and PARV4 genotype 2, BR10627-5 (GenBank accession no. DQ873390) and C25-5 (GenBank accession no. DQ873391). The PARV4 original strain was sourced from the index case patient in 2004 [1]. Strains BR10749-4 and BR10627-5 were identified in our preliminary study of plasma pools [8], while the other strains were identified in further screening studies of manufacturing plasma pools [9]. Strains BR10749-4, BR11955-4 and BR10627-5 were from plasma samples received at NIBSC between 2004 and 2005, while A23-4, C51-4 and C25-5 were received at NIBSC 1990–1993. The alignment also includes the corresponding nucleotide sequences of porcine parvovirus 2 (PPV-2) (GenBank accession no. AB076669) and B19V-Au genotype 1 virus (GenBank accession no. M13178) as outgroups. Genetic distance and bootstrap values are indicated.

