B19 infectivity and immunity merits consideration. In the present article, we describe the serological analysis of specimens obtained from 10 individuals who participated in the postmarketing study [3, 8]; this analysis extends our knowledge of the immune response to B19 exposure.

Materials and methods. As part of a postmarketing study, 100 adult volunteers, previously determined to be B19 IgG$^+$ by use of an *Escherichia coli*–based EIA to detect B19 IgG, were each transfused with 1 unit (200 mL) of pooled plasma (Plas+SD) [3, 8, 10]. Paired plasma specimens (blinded) were obtained pretransfusion and 1 month posttransfusion from 10 of the volunteers.

The 20 plasma specimens were analyzed for both B19 IgM and B19 IgG reactivity against capsid (conformational) VP2 (VP2-N), by use of FDA-approved EIAs (Biotrin). B19 IgG levels were quantified using the World Health Organization B19 IgG International Standard (93/724) [11]. Furthermore, B19 IgG reactivity against conformational (N) and linear (D) epitopes on VP1 (VP1-N and VP1-D, respectively) and to linear VP2 (VP2-D) was analyzed as described elsewhere [12]. The subsequent classification of pooled-plasma recipients into groups I, II, and III, as well as the details of plasma pools used for transfusion, is shown in table 1.

Results. Figure 1*A* shows that specimens from groups I and II contained no VP2-specific IgM reactivity, whereas specimens from group III exhibited high levels of B19 IgM reactivity posttransfusion, thereby confirming acute B19 infection in this cohort. Analysis of the B19 IgG reactivity of individual plasma specimens was performed both pre- and posttransfusion, and 3 specimens (01002, 01052, and 01098) of 10 exhibited reactivity against VP2-N pretransfusion, with the range of B19 IgG in these specimens being 19–39 IU/mL (figure 1*B*, group I); there was a subsequent increase in the level of B19 IgG reactivity posttransfusion, which resulted in 2 of 3 specimens (both transfused with plasma pool PS3 [table 1]) exhibiting B19 IgG levels >100 IU/mL and the third specimen exhibiting an increase to 50 IU/mL.

A further 3 specimens (01023, 01053, and 01055), 2 of which were from individuals transfused with plasma pool PS2A (table 1), were seronegative for antibodies against VP2-N (B19 IgG <3 IU/mL), both pre- and posttransfusion (figure 1*B*, group II).

The remaining 4 paired pretransfusion specimens tested contained no detectable B19 IgG against VP2-N; however, the corresponding paired posttransfusion specimens exhibited evidence of B19 seroconversion and exhibited high levels of reactivity against VP2-N epitopes (figure 1*B*, group III); this reactivity corresponded to B19 IgG levels >100 IU/mL in 2 of 4 of the specimens, whereas the remaining 2 specimens contained lower levels of B19 IgG, equivalent to 50 and 78 IU/mL, respectively. For each specimen, the pattern of reactivity against VP1-N epitopes was identical to that exhibited against VP2-N, whereby IgG specific for VP1-N was increased posttransfusion in group I and was also evident only posttransfusion in group III (figure 1*C*).

When specimens were analyzed for reactivity against VP1-D epitopes, 2 specimens (01002 and 01098) of 3 from group I did not exhibit significant pretransfusion IgG reactivity; however, these 2 specimens did display significant posttransfusion antibody reactivity (mean ± SD IgG index value, 3.5 ± 1.7 [IgG index value >1.1 is reactive]) (figure 1*D*). The remaining specimen (01052) was seronegative for VP1-D IgG, both pre- and posttransfusion. Group II specimens were unreactive against VP1-D. All group III specimens were seronegative for B19 VP1-D IgG pretransfusion; posttransfusion, however, all had high levels of antibody reactivity against VP1-D epitopes (mean ± SD IgG index value, 3.9 ± 0.96).

B19 IgG reactivity was observed only against VP2-D epitopes in group III specimens, with a mean ± SD VP2-D IgG index value of 4.5 ± 1.8 (figure 1*E*). It should be noted that, although posttransfusion group I specimens exhibited an increase in levels of B19 IgG against VP2-N epitopes (figure 1*B*), they had no increase in antibody reactivity to VP2-D epitopes.

Discussion. The present study demonstrates that, in B19-seropositive recipients transfused with plasma containing high levels of B19 DNA (1.6×10^8 IU/mL), levels of parvovirus B19 IgG against VP1-N and VP2-N epitopes and against linear

Table 1. Classification of pooled-plasma recipients, according to B19 IgG reactivity against conformational epitopes on B19 VP2.

Group no., pooled-plasma–recipient code no.	Plasma pool transfused	B19 DNA level
Group I		
01002	PS3	1.6×10^8 IU/mL
01052	NA	NA
01098	PS3	1.6×10^8 IU/mL
Group II		
01023	PS2A	$10^{3.5}$ GE/mL
01053	PS2A	$10^{3.5}$ GE/mL
01055	NA	NA
Group III		
01005	PS1	2.2×10^8 IU/mL
01048	PS1	2.2×10^8 IU/mL
01057	PS1	2.2×10^8 IU/mL
01069	PS3	1.6×10^8 IU/mL

NOTE. Plasma pools PS1 and PS3 contained 59.5 and 72.0 IU of B19 IgG/mL, respectively [9]. The level of B19 DNA in plasma pool PS2A was provided by A. Lazo. Group I and II recipients remained symptom free, whereas group III recipients experienced mild fever and malaise, after transfusion. Group I, recipients who were seropositive before transfusion ($n = 3$); group II, recipients who were seronegative both before and after transfusion ($n = 3$); group III, recipients who were seronegative before transfusion and seropositive after transfusion ($n = 4$); GE, genome equivalent; NA, not available.

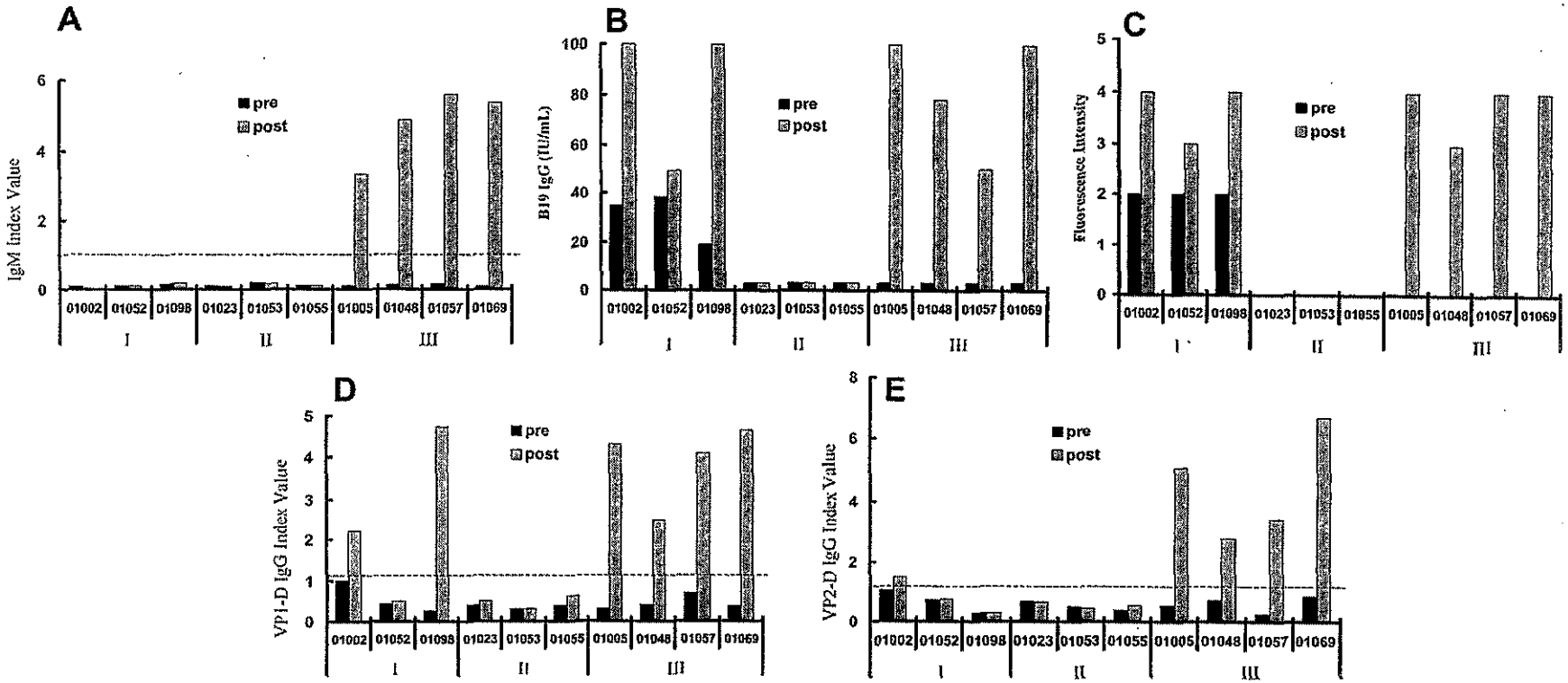

Figure 1. *A*, B19 IgM reactivity against conformational VP2, in pooled-plasma recipients (before and after transfusion). Reactivity is measured as IgM index value (reactivity >1.1 is reactive *[dashed line]*). *B*, B19 IgG reactivity against capsid (conformational) VP2 (VP2-N), as determined by EIA and expressed as IU/mL, in pooled-plasma recipients. *C*, B19 IgG reactivity against conformational epitopes on VP1 (VP1-N), in pooled-plasma recipients. Reactivity is assessed using an immunofluorescence assay and is graded, according to the manufacturer's instructions on a scale of 1–4, depending on the extent of fluorescence. *D*, B19 IgG reactivity against linear VP1 (VP1-D), in pooled-plasma recipients (before and after transfusion). *E*, B19 IgG reactivity against linear VP2 (VP2-D), in pooled-plasma recipients. *D* and *E*, Reactivity measured as IgG index value (index value is specimen:cutoff OD ratio; reactivity >1.1 is reactive *[dashed line]*).

epitopes on the unique region of VP1 increase dramatically. Moreover, it also proposes that the levels of B19 IgG in pooled-plasma products protect against infection in B19-seronegative recipients when only low levels of B19 DNA (i.e., $<10^{3.5}$ GE/mL) are present. Finally, we have shown that, in the presence of $1.6–2.2 \times 10^8$ IU of B19 DNA/mL, B19 IgG levels of 59.5 (plasma pool PS1) and 72.0 IU/mL (plasma pool PS3), respectively, are insufficient to prevent B19 transmission to B19-seronegative recipients (group III) and subsequent seroconversion.

Group I recipients were seropositive for B19 IgG before transfusion with pooled plasma. The level of IgG specific for VP2-N increased to >100 IU/mL in 2 recipients after transfusion with plasma pool PS3; however, the observed increase in the remaining recipient (01052) was lower (50 IU/mL). This subsequent increase in IgG response was mirrored by the increased reactivity against VP1-N that was observed posttransfusion, whereby the increase in fluorescence exhibited by the specimen from recipient 01052 was less than that for the others in group I. It is relevant that, because of the presence of high-titer B19 DNA, blood products lacking B19-specific antibodies were most at risk of transmitting B19 infection and that, despite high levels of B19 DNA, recipients with preexisting B19 IgG (or who were the administered blood products containing B19 IgG) were not infected [13]. Plentz et al. [14] have also confirmed that the presence of B19 IgG in either the recipients of the blood products or in the administered material offers protection against B19 DNA (at concentrations of $<6 \times 10^2–2.2 \times 10^6$ GE/mL) present in therapeutic products, to the extent that no individual ($n = 14$) receiving a B19-contaminated blood product showed symptoms of acute B19 infection. The results of the present study demonstrate that, in a healthy immunocompetent individual (recipient 01098), a B19 IgG level of 19 IU/mL confers protection against the development of symptoms of B19 infection when that individual is reexposed to the virus. To our knowledge, the present study is the first to demonstrate that there is a specific level of B19 IgG that protects against reinfection. The postexposure B19 IgG profile will also contribute to avoidance of reinfection.

Although all recipients in group I had either lost or never developed antibody reactivity against VP1 or VP2 epitopes before transfusion, 2 of them subsequently displayed strong IgG responses against linear epitopes on the VP1-unique region only and not against VP2-D. This observation is in accordance with the work of Soderlund et al. [15] and significantly strengthens our hypothesis that VP1-specific B-cell memory is maintained only with respect to linear epitopes of the unique region of VP1, as well as with respect to VP1-N/VP2-N epitopes [12]. Recipient 01052 in group I exhibited the lowest increase in B19 IgG reactivity after transfusion and was seronegative for antibody reactivity against VP1-D both before and after transfusion, possibly as a result of infusion with plasma containing a B19 viral load lower than that required for reactivation of the memory response.

Group II recipients all remained seronegative after receipt of pooled plasma. Although information was not available on which plasma pool was transfused into recipient 01055, both recipient 01023 and recipient 01053 were transfused with plasma pool PS2A, which contained $10^{3.5}$ B19 GE/mL [3]. Given that the mean level of B19 IgG observed in pooled plasma is 64.7 ± 17.5 IU/mL [9], it is clear that B19 IgG within this range appears to be protective against infection of seronegative recipients when the B19 viral load is $\leq 10^{3.5}$ B19 GE/mL.

Group III recipients who underwent B19 seroconversion after transfusion exhibited both strong VP2-specific IgM reactivity and significant levels of B19 IgG against VP2-D epitopes. The latter result is in accordance with previously published findings that production of antibody directed against VP2-D epitopes occurs shortly after exposure to B19 [15].

Traditionally, plasma-product manufacturers have relied on the presence of high levels of B19 IgG in pooled-plasma products alone to indicate product safety [2]. The data presented in the present study reinforce the strategy of identifying and removing high-titer B19 plasma donations from plasma pools, given that 4 of 7 recipients seroconverted because of the presence of B19 DNA in solvent/detergent-treated pooled human plasma. Although many companies have introduced minipool screening to address this problem, such screening is not presently mandatory, despite the fact that it is usually high-risk populations (e.g., pregnant women and immunocompromised patients) who are administered such products. This regulatory ambiguity is likely to change in coming years, as improved product-safety profiles are demanded by consumers.

In summary, the present study has provided new data relevant to the B19 IgG level necessary to confer protection after reexposure to the virus, as well as to the B19 IgG level that, in pooled-plasma products, may prevent infection of seronegative recipients.

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識別番号・報告回数			報告日	第一報入手日 2006年7月10日	新医薬品等の区分 該当なし	厚生労働省処理欄

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販売名（企業名）	①献血ヴェノグロブリン-IH ヨシトミ（ベネシス） ②ヴェノグロブリン-IH（ベネシス） ③グロブリン-Wf（ベネシス）			

研究報告の概要

ヒトパルボウイルスB19 DNAは、しばしば血漿由来凝固因子製剤に検出される。加えてB19感染の伝播が観察され、製造工程中に日常的に行われるウイルスの不活化/除去の工程にもかかわらず、感染性ウイルスが存在していることを示している。最近、ヒトパルボウイルスB19の分類が3つの異なる遺伝型に分かれることが確認された。これまで、凝固因子製剤の遺伝子型2による汚染の情報はない。このため、我々はPCRにより、遺伝子型1及び2について、202の異なる凝固因子製剤のロットを調査した。最近3年間に投与された13の異なる製品の181のロットについて分析を行い、1980年代初めまで使用されたウイルス不活化処理のされていなかった21ロット（8製品）と比較した。遺伝子型1 DNAが、現在投与されているロットの77/181（42.5%）に、以前に使用されたロットの17/21（81%）に検出された。遺伝子型2 DNAは、5/202（2.5%）に見出され、その5ロット全てが、遺伝子型1 DNAにも汚染されていた。遺伝子型2 DNAが見出された5ロットは、血液凝固第VIII因子製剤の5ロットで、現在使用されているロットで2ロット、1980年代初めまで使用されていたロットで3ロットが含まれていた。
DNA配列分析は、PCRで2重に陽性であった製剤は、典型的な遺伝子型1と遺伝子型2のDNAを含んでいることを示していた。遺伝子型2は遺伝子型1と類似の疾患スペクトラムを起こすように見えることから、現在プール血漿に広く適用されている遺伝子型1に加えてNATでの遺伝子型2の同時検出によって、血液製剤の安全性のレベルを引き上げることができるであろう。

使用上の注意記載状況・その他参考事項等

代表として献血ヴェノグロブリン-IH ヨシトミの記載を示す。
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。
3. 略
4. 略
5. 略
6. 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。〕

報告企業の意見	今後の対応
ヒトパルボウイルスB19の変異型である遺伝型2が血液凝固第VIII因子製剤から検出された報告である。 弊社が最終製剤の試験に用いているキットは医学生物学研究所製「スマイテストパルボウイルスB19遺伝子定性キット」であり、このキットは、パルボウイルスB19遺伝型2についても検出可能であることを確認している。万一、原料血漿にパルボウイルスB19遺伝型2が混入したとしても、CPVをモデルウイルスとしたウイルスバリデーション試験成績およびパルボウイルスB19を用いた不活化・除去試験結果から、本剤の製造工程において十分に不活化・除去されると考えている。	本報告は本剤の安全性に影響を与えないと考えるので、特段の措置はとらない。

Contamination of coagulation factor concentrates with human parvovirus B19 genotype 1 and 2

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Summary

Human parvovirus B19 (B19) DNA has frequently been detected in plasma-derived coagulation factor concentrates. Furthermore, transmission of B19 infection was observed, indicating presence of the infectious virus despite routine viral inactivation/removal procedures during the manufacturing process. Recently, human parvovirus DNA isolates, variant from B19, have been identified resulting in classification of B19 virus into three distinct genotypes, with all viruses previously classified as B19 belonging to genotype 1. So far, there is no information available on contamination of clotting factor concentrates with genotype 2. Therefore, we analysed 202 different factor concentrate lots for genotype 1 and 2 DNA by PCR. Analysis of one hundred eighty-one lots representing 13 different products, administered over the last three years, was compared to 21 lots (8 products) used until the early 1980s which had not been treated by viral inactivation procedures. Genotype 1 DNA was detected in 77/181 (42.5%) currently administered lots, and 17/21 (81%) previously used lots. The level of genotype 1 DNA contamination was similar in currently and previously administered concentrates. Genotype 2 DNA was found in 5/202 (2.5%) lots, all of which were co-contaminated with genotype 1 DNA. DNA sequence analysis showed that the PCR-double positive concentrates contained typical genotype 1 and genotype 2 DNA. Because genotype 2 appears to cause a similar spectrum of diseases as genotype 1, simultaneous detection of genotype 2 by nucleic acid amplification testing (NAT), now widely applied to plasma pools for genotype 1, would give an added level of safety to blood products.

Keywords

Human parvovirus, genotype 2, coagulation factor concentrates

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Introduction

Human parvovirus B19 (B19) DNA has frequently been detected in plasma-derived coagulation factor concentrates (1-5). Transmission of B19 infection by derivatives produced from pooled plasma has been reported thus indicating the presence of the infectious virus despite routine viral inactivation/removal procedures during the manufacturing process (6-12). However, the effectiveness against B19 of the current inactivation procedures is unclear due to physical robustness of the nonenveloped virus.

Recently, it has been shown that the genetic diversity of B19 virus is higher than previously expected (13-15; for review 16). Therefore, the species B19 is now subdivided into three different genotypes, with all viruses previously classified as B19 belonging to genotype 1. Until now, genotype 2 DNA has been detected in several European countries including Germany, and the United States (14, 17, 18), whereas detection of B19 genotype 3 was mainly limited to France (15). According to current data, genotype 2 and genotype 3 viral infections cause the same spectrum of illnesses as "classical" B19 infections (15). Given the relatively high homology between the viral proteins, sero-

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logical cross-reaction and a certain degree of cross-protection between the three genotypes could be expected, at least in immunocompetent individuals.

Since there is no information on the frequency of contamination with B19 genotype 2, we investigated, by nested polymerase chain reaction, 202 lots of coagulation factor concentrates representing 21 commercially available products for both genotype 2 and genotype 1 DNA. One hundred and eighty-one lots (13 different products) were currently used concentrates administered during the last three years and 21 lots (8 different products) were formerly used concentrates administered up to the beginning of the 1980s and not virally inactivated. In case of a positive genotype 2 result, DNA sequence analysis was performed. The viral load of genotype 1 DNA in currently and formerly administered concentrates was quantitatively measured by real-time polymerase chain reaction.

Table 1: Detection of parvovirus B19 genotype 1 and genotype 2 DNA in currently administered coagulation factor concentrates.

Coagulation factor	Product	Virus inactivation	IU[1]	No. of lots tested	PCR results: No. of positive lots (%) Genotype 1	Genotype 2
Factor VIII	A	S/D & dry heat 80 °C 72 h	1000	35	7	0
			500	7	4	0
				Σ 42	11	0
	B	Tween 80 & vapour heat 60 °C 10 h	1000	16	12	0
			500	8	4	0
			250	4	0	0
				Σ 28	16	0
	C	S/D & dry heat 100 °C 0.5 h	1000	25	20	2*
			500	2	0	0
				Σ 27	20	2
	D	S/D	1000	11	3	0
	E	Pasteurisation 60 °C 10 h	1000	7	1	0
			500	2	0	0
				Σ 9	1	0
	F	Pasteurisation 60 °C 10 h	1000	10	7	0
	G	S/D & dry heat 80 °C 72 h	1000	1	1	0
	H	S/D & dry heat 100 °C 0.5 h	250	1	1	0
				Total 129	60 (46.5%)	2 (1.6%)
Factor IX	I	Tween 80 & vapour heat 60 °C 10 h, 80 °C 1 h	1000	14	5	0
			600	4	2	0
				Σ 18	7	0
	J	S/D & nanofiltration	1000	10	4	0
			500	3	1	0
				Σ 13	5	0
	K	S/D	500	2	0	0
				Total 33	12 (36.4%)	0
Factor VII	L	Vapour heat 60 °C 10 h, 80 °C 1 h	500	2	0	0
Act. prothrombin complex concentrate	M	Vapour heat 60 °C 10 h, 80 °C 1 h	1000	8	3	0
			500	9	2	0
				Total 17	5	0
All products				Total 181	77 (42.5%)	2 (1.1%)

[1] IU = International Units coagulation factor per vial
* Genotype 2 DNA positive lots referred to as C_1 and C_2

Materials and methods

Coagulation factor concentrates

In total, 202 lots of 21 commercially available plasma-derived coagulation factor concentrates were investigated. One hundred and eighty-one lots (13 different products) were currently available concentrates, administered during the last three years (specimens collected between October 18, 2000 and February 28, 2003), compared to 21 lots (8 different products) taken from formerly used concentrates, administered until the beginning of the eighties and not virally inactivated. Details of the investigated coagulation factor concentrates are given in Table 1 and Table 2.

DNA isolation and polymerase chain reaction

DNA was prepared from 200 µl of reconstituted factor by spin column procedure (QIAamp DNA® Mini Kit, Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Nested polymerase chain reaction (PCR) for detection of B19 genotype 1 DNA was performed as described previously (19). The assay was found to reliably detect the presence of 2 genome equivalents per reaction. PCR was specific for genotype 1 DNA. Amplification of genotype 2 DNA was performed using the primers described by Hokynar et al. (14). PCR was carried out in 50 µl volumes with the following concentration of reagents: 250 µM of each deoxynucleoside triphosphate (Ultrapure dNTPs, Amersham Biosciences, Freiburg, Germany), 25 pmol of each primer (Sigma-Genosys, Steinheim, Germany), 5 µl 10× PCR-buffer (Expand High Fidelity PCR System, Roche Diagnostics, Mannheim, Germany), 2 mM $MgCl_2$ (Roche Diagnostics) and 1.75 U DNA polymerase (Expand High Fidelity PCR System, Roche), and 5 µl of DNA preparation. From first round reaction mixture, 5 µL were transferred to the second round reaction mixture containing the same constituents as the first round mix, except for the nested primers. Amplification was as follows: 95 °C for 5 min followed by 35 cycles each consisting of 94°C for 10 s, 50°C for 10 s, and 72°C for 20 s (T3 Thermocycler, Biometra®, Göttingen, Germany). A final elongation step followed for 3 min at 72°C. Identical conditions were used for the first and second round amplification. The assay accurately detects 2 genome equivalents per sample, determined as mentioned below. Figure 1 illustrates the positions of the amplified regions in the B19 genome.

10 µl of the second-round PCR mixture were analysed by electrophoresis on agarose composite minigels of 1.5%NuSieve® GTG® (FMC)/0.5%SeaKem® LE (FMC) (Cambrex, supplied by Biozym, Hessisch Oldendorf, Germany). Amplified products were visualised by ethidium bromide staining and UV illumination. Positive and negative controls were included in every run. For negative control, all PCR reagents and sterile bidistilled water instead of the sample was used. Strict precautions to avoid contaminations were taken.

DNA sequence analysis

For DNA sequence analysis, half of the genome was amplified by nested PCR using genotype-specific oligonucleotide primers (Fig. 1). For sequencing of genotype 1 DNA, all four nested primer pairs described by Hemauer et al. (20; amplification regions: NS1-C, ΔV, VP1/VP2 and VPC) were used. Additionally, primers for amplification of the genome region between amplification regions VP1/VP2 and VPC (region VPint) were used: outer forward 5′-ACAATGCCAGTGGAAAGGAG-3′ (nucleotide (nt) 3318-3337; all positions according to B19 genotype 1 strain Au, GenBank accession no. M13178) (21); outer reverse 5′-CCCAGGGCGTAAGGA-

Table 2: Detection of parvovirus B19 genotype 1 and genotype 2 DNA in coagulation factor concentrates administered until the beginning of the 1980s.

Coagulation factor	Product	IU[§]	No. of lots tested	PCR results: No. of positive lots (%)	
				Genotype 1	Genotype 2
Factor VIII	a	250-1100	8	6	0
	b	250 / 500	4	4	1*
	c	250 / 500 / 1000	4	2	0
	d	1000	1	1	1
	e	250	1	1	0
	f	1000	1	1	0
	g	500	1	1	1
	h	1000	1	1	0
			Σ 21	17 (81%)	3 (14%)

§ IU = International Units coagulation factor per vial
* Lot contains 500 IU

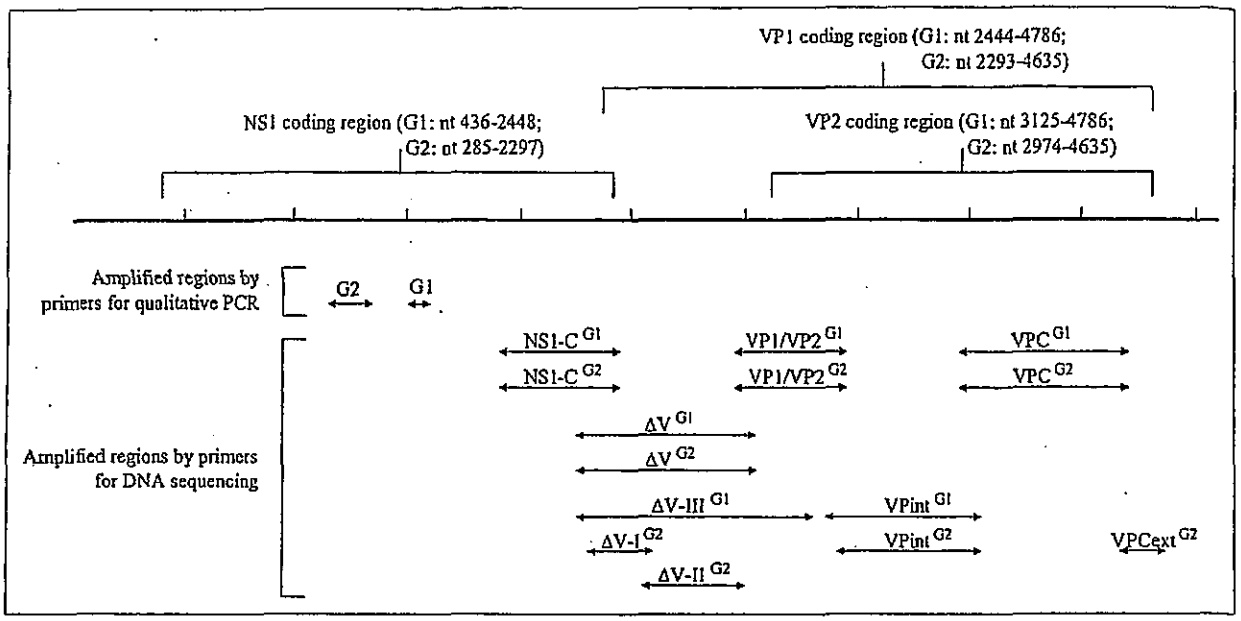

Figure 1: Schematic representation of the amplified regions of the parvovirus B19 genome. The reading frames of the viral proteins NS1, VP1 and VP2 are indicated in the upper part of the figure. Numbering of the nucleotides of B19 genotype 1 (G1) according to the genotype 1 strain Au (GenBank accession no. M13178); numbering of the nucleotides of genotype 2 (G2) according to strain LaLi (GenBank accession no. AY044266). The genome regions amplified by nested PCR using genotype 1 (G1) and genotype 2 (G2) -specific primers are shown in the lower part of the figure. G1- and G2-specific primers used for qualitative PCR were previously described (14, 19); G1- and G2-specific primers for DNA sequencing as mentioned in *material and methods*.

TATT-3′ (nt 4117-4099); nested forward 5′-AAGGTTTGCACCATCAGTCC-3′ (nt 3341-3360); nested reverse 5′-TTAAGGCTTTTCCAGCTCCA-3′ (nt 4064-4045). In cases where no PCR product was obtained by ΔV-specific primers, the following primer set was used (amplification region ΔV-III): outer forward 5′-CTACACACCTTTGGCAGACC-3′ (nt 2151-2170); outer reverse 5′-GGACTGATGGTGCAAACCTT-3′ (nt 3360-3341); nested forward 5′-TTTACCTGTGTGTTGTGTGCAA-3′ (nt 2223-2244); nested reverse 5′-CTGCGGGAGAAAACACCTTA-3′ (nt 3305-3286).

For amplification of genotype 2 DNA the sequences of the primers used for the genotype 1 amplification regions NS1-C, ΔV, VP1/VP2 and VPC were modified according to the sequence of the genotype 2 strain LaLi (14; GenBank accession no. AY044266). Primers for amplification of the genome region between amplification region VP1/VP2 and VPC (amplification region VPint) were as follows: outer forward 5′-CAGTGGAAAAGAGGCAAAGG-3′ (nt 3174-3193; nucleotide positions according to genotype 2 strain LaLi; note that homologous genome regions of genotype 1 and 2 are not congruently numbered); outer reverse 5′-CCAGTGATGGTATGGCTGTG-3′ (nt 3993-3974); nested forward 5′-CATAATGGGCTACTCAACACCA-3′ (nt 3210-3231); nested reverse 5′-GCGCCTGTATTGGAAGTGTC-3′ (nt 3899-3880). When the modified primers failed to amplify the region ΔV the following primer sets were used: amplification region ΔV-I: outer forward 5′-ATTGCCTGTTTGTTGTGTGC-3′ (nt 2072-2091); outer reverse 5′-ATAGGTCTGGAGAGTCTTTAAGATTAC-3′ (nt 2521-2495); nested forward 5′-TGTCCTCATTGTATTAATGTGGGA-3′ (nt 2127-2150); nested reverse 5′-CAAACAGGGAAGATGGGTTT-3′ (nt 2473-2454); amplification region ΔV-II: outer forward 5′-AGGATGTGTATAAGCAATTTGTA-3′ (nt 2342-2364); outer reverse 5′-CTTTTCTGAGGCGTTGTATGC-3′ (nt 2964-2944); nested forward 5′-GTTACTGGGACAGACTTAGAGCTTATA-3′ (nt 2380-2406); nested reverse 5′-ATCTTTTACTGCTTGTGCTTGAA-3′ (nt 2877-2855). For amplification of the region encoding the extreme C-terminal region of the viral structural proteins, the following primers were used (amplification region: VPCext): outer forward 5′-TGGACCAATTGGGGGTATTA-3′ (nt 4315-4334; positions according to genotype 2 strain A6 clone c2, GenBank accession no. AY064475 (17); corresponding sequences of strain LaLi not available); outer reverse 5′-GTTCTCTGCGGGGTATTGG-3′ (nt 4683-4665); nested forward 5′-GAATCCACAGCCTGGAGTGT-3′ (nt 4432-4451); nested reverse 5′-TCTGGGTGGTACAGGAGGAC-3′ (nt 4649-4630).

For DNA sequencing, nested PCR products were purified by QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequencing reactions were carried out using approximately 5-20 ng of the purified PCR product. Amplicons from at least two independent PCR reactions were sequenced in the forward and