別紙様式第2-1

No. 10

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販売名(企業名)	合成血「日赤」(日本赤十字社) 照射合成血「日赤」(日本赤十字社)			

研究報告の概要

○献血適格者におけるヒトパルボウイルスB19の持続感染

ヒトパルボウイルスB19持続感染の自然経過の特徴を明らかにするための長期的研究を実施した。日本では、全ての献血血液にRHA法によるB19抗原検査を行っている。この方法を用いて、1997年～1999年に大阪の献血者979,052人からB19感染102例を特定した。102名のうち、次の献血に訪れた20名(男性15名、女性5名;平均年齢34.3歳)から血漿検体を採取し、ウイルス力価及びB19 IgG・IgM抗体力価を測定することができた。B19 DNAについてはTaqMan PCR法、B19抗体については酵素免疫測定法を使用した。B19抗原陽性の血液は不適として廃棄されるため、初回献血時についてはこれらの検査を行っていない。平均フォローアップ期間は838日(範囲、101～1749日)だった。

血漿B19 DNAは、最初の6ヶ月間で急速に減少し、その後も減少は続いたが検出不能にはならなかった。B19抗体については、IgG、IgM両方が検出された9名ではIgMが検出不能となったが、他の9名ではIgGのみが検出され、IgMは2度目の献血の前に検出限界以下まで低くなったと考えられた。残り2名の献血者は調査期間の最後までIgMが検出可能だった(729日、743日)。B19抗体の当初の分析結果は異なるパターンを示したが、一度感染が成立すると、B19 IgGは20人の献血者全員で持続した。

これまでの研究結果と同様、本長期研究において献血者のB19持続感染が観察された。フォローアップ期間中、20名の献血者は高値のB19 IgGと低いウイルス力価を維持していたが、B19感染の症状を報告した者はいなかった。本研究のデータは、B19急性感染後の血漿ウイルス力価は約1年で10^4IU/mL未満、約2年で10^3IU/mL未満まで下がることを示された。ここで観察されたウイルス力価の動態は、B19 NATが実施できない状況において、より適切な献血者の選択に役立つだろう。これらの予備的な知見を実証するために、より大規模な研究が望まれる。

報告企業の意見	今後の対応
大阪のヒトパルボウイルスB19陽性献血者20名のB19 DNA、IgG・IgM抗体を長期間フォローアップしたところ、B19持続感染が観察されたが、B19感染の症状を報告した者はいなかった。B19急性感染後の血漿ウイルス力価は約1年で10^4IU/mL未満、約2年で10^3IU/mL未満まで下がることが示されたとの報告である。	今後も引き続き、ヒトパルボウイルスB19に関する新たな知見及び情報の収集に努める。日本赤十字社では、以前よりRHA法によるB19抗原検査を導入、ウイルス量の多い血液を排除している。また、2008年には感度向上のため検査法をCLEIA法に変更した。

使用上の注意記載状況・その他参考事項等

合成血「日赤」
照射合成血「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

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LETTERS TO THE EDITOR

Persistent infection by human parvovirus B19 in qualified blood donors

Persistent parvovirus B19 infection with a low viral load has been reported in immunocompromised and in immunocompetent individuals (reviewed in Parsyan and Candotti[1]). Large cross-sectional studies using highly sensitive DNA amplification methods have also demonstrated persistent B19 infection.[2] Recently, Lefere and colleagues[3] conducted a longitudinal study of nonimmunodeficient patients who were multitransfused with red blood cells, demonstrating that asymptomatic chronic B19 infections may persist for a long period.[3] To characterize the natural course of persistent B19 infections, we conducted the following longitudinal study using an in-house TaqMan polymerase chain reaction method for B19 DNA and enzyme immunoassays to detect B19 immunoglobulin M (IgM) and immunoglobulin G (IgG; Denka Seiken, Tokyo, Japan). This study was approved by the ethical Committee of the Japanese Red Cross Osaka Blood Center. In Japan, all donated blood is tested for B19 infection with an in-house receptor-mediated hemagglutination method that detects B19 antigen as a marker of a high viremic stage of infection (cutoff, approx. 2.5×10^{10} IU/mL B19 DNA; data not shown). Using this method, we identified 102 cases of B19 infection among 979,052 blood donors in Osaka between 1997 and 1999. We were able to test the plasma samples of 20 of these 102 donors (15 male, 5 female; mean age, 34.3 years) when they returned for subsequent blood donations for viral load and B19-specific IgG and IgM. We did not examine the donors at their first visit because B19 antigen-positive blood was automatically disqualified and disposed. The mean duration of follow-up was 838 days (range, 101-1749 days). The results of sequential viral load testing for all donors are shown in Fig. 1A. In the first 6 months, we observed a rapid decline in plasma B19 DNA, which decreased continuously, but never became undetectable. Median plasma B19 viral loads for samples tested within every 6 months are shown in Fig. 1B. We analyzed the B19 antibody for all donors during the study period (Fig. 2A). For 9 donors (Donors 1-9) with both IgG and IgM, IgM became undetectable, while for 9 others (Donors 10-18), only B19 IgG was detected, presumably because B19 IgM had decreased to an undetectable level before the second visit. The remaining 2 donors (Donors 19 and 20) had B19 IgM–detectable until the last visit (at 729 and 743 days). Although the initial profile for B19 antibodies showed different patterns, once established, B19-specific IgG persisted in all 20 donors. Summaries for 3 representative cases corresponding to each of these patterns for IgM, IgG, and viral load are presented in Fig. 2B.

Consistent with previous studies that suggest that B19 DNA may persist for a long period in immunocompetent individuals,[3-5] we observed persistent B19 infection in healthy blood donors in the present longitudinal study. During the follow-up period, none of the 20 infected blood donors reported symptoms of B19 infection, although they retained high levels of B19 IgG and low viral load. Our data suggest that in healthy individuals, the B19 plasma viral load declines to below 10^4 IU per mL in approximately 1 year and to 10^3 IU per mL in approximately 2 years after an acute (high viremia) infection. The patterns of plasma B19 viral load observed in our study may be useful for identifying more suitable blood donors in circumstances where B19 NAT is unavailable. We encourage further studies with a larger sample size to validate these preliminary findings.

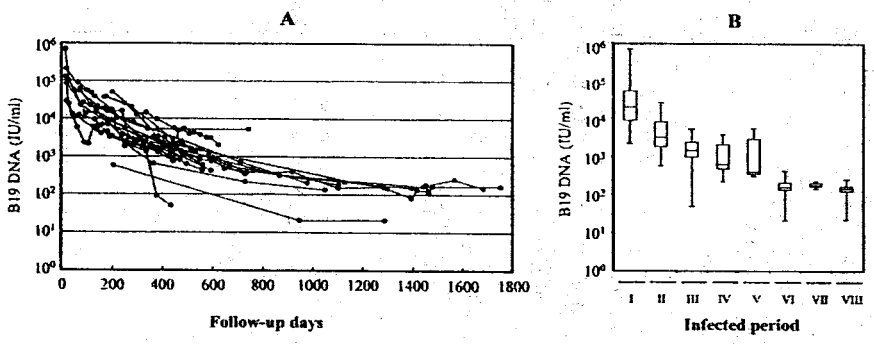

Fig. 1. (A) Changes in plasma B19 viral load in healthy blood donors after an acute B19 infection. Each line represents plasma B19 DNA of the same donor. Time 0 was defined as the first test visit when positive results were obtained for B19 antigen (high viremic phase). (B) Plasma B19 viral loads for all cases by 6-month intervals. Medians of the plasma B19 viral load with its 75th (top of the box) and 25th (bottom of the box) percentiles in each category were indicated. I = 0 to 0.5 years (0-182 days); II = 0.5 to 1.0 years (183-365 days); III = 1.0 to 1.5 years (366-549 days); IV = 1.5 to 2.0 years (550-730 days); V = 2.0 to 2.5 years (731-914 days); VI = 2.5-3.0 years (915-1096 days); VII = 3.0-3.5 years (1096-1279 days); VIII = at least 3.5 years (≥1280 days).

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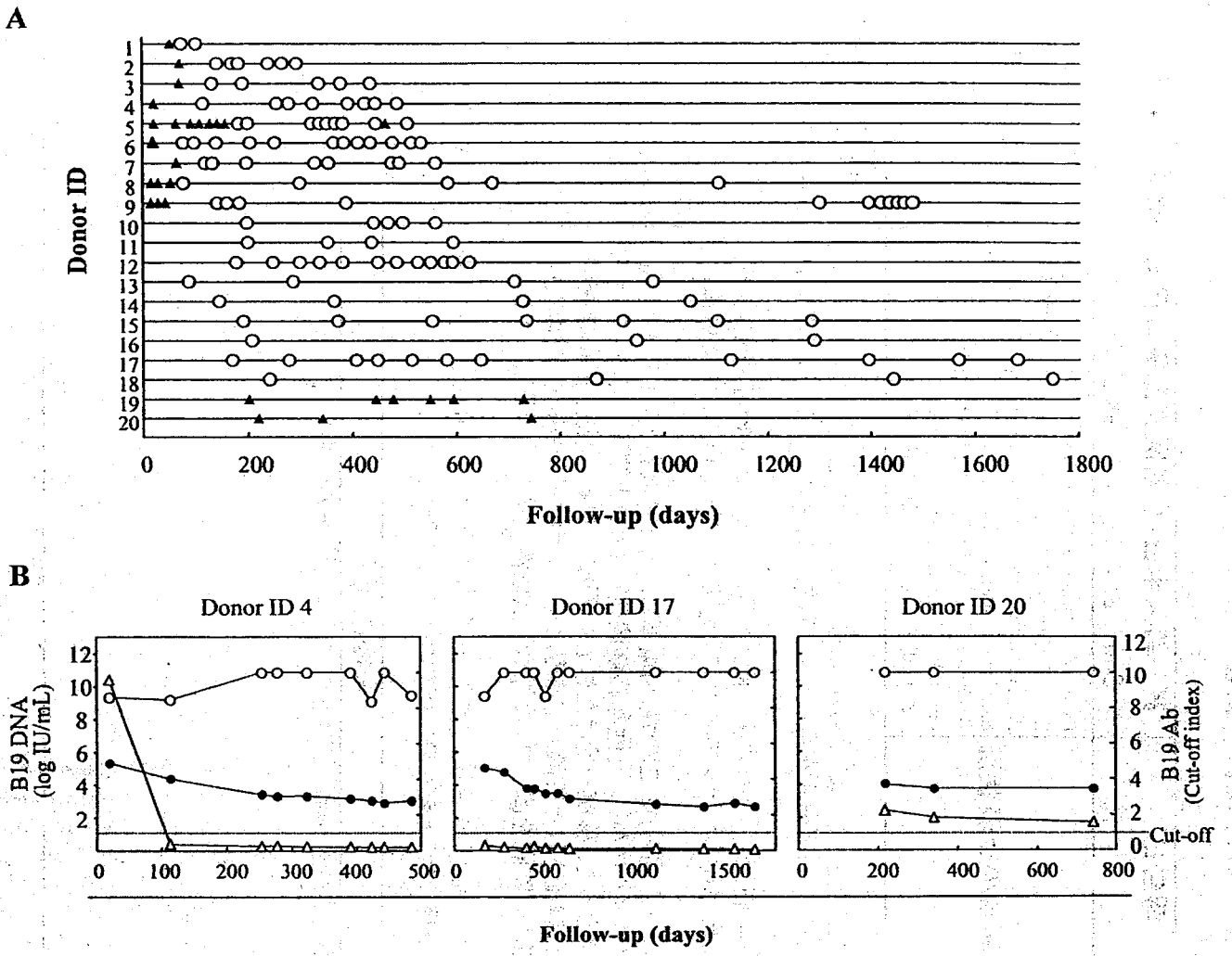

Fig. 2. (A) B19 IgM and IgG for individual donors at follow-up visits. (▲) Positive for both IgM and IgG; (○) positive for IgG. (B) Representative cases for three patterns of test results. Changes in viral load (●), IgM (△), and IgG (⊙). Donors correspond to those in A.

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New cell lines express HNA-1c, -4a, -4b, -5a, or -5b for identification of HNA antibodies

Antibodies to human leukocyte antigens (HLAs) or human neutrophil antigens (HNAs) are regarded to be the principal causes of nonhemolytic transfusion reactions, including transfusion-related acute lung injury. Although flow cytometric (FCM) analysis using panels of phenotyped neutrophils is widely used to detect and identify antibodies to HNAs, FCM is time-consuming and

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医薬品
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別紙 3-3

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2008 年 4 月 16 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Inactivation of parvovirus B19 during STIM-4 vapor heat treatment of three coagulation factor concentrates Berting, A. et al, Transfusion, ahead of print	公表国 オーストリア	
販売名（企業名）						

研究報告の概要	使用上の注意記載状況・その他参考事項等
ヒトパルボウイルス B19（B19V）の感染性アッセイが可能となる以前は，モデルウイルスと呼ばれる動物パルボウイルス(例：マウスパルボウイルス)が，血漿タンパクの製造工程におけるウイルス不活化の確認に使用されており，一般的にパルボウイルスは熱不活化に耐性があると考えられていた。しかし，最近の知見より，B19V は動物パルボウイルスよりも熱に弱いことが明らかになってきた。 本文献は，数種の血液凝固因子製剤において STIM-4 蒸気加熱処理装置を用いた不活化処理を行い，B19V とモデルウイルスとして用いられていたマウス微小ウイルス（MMV）間での不活化効果の比較を行っている。 血液凝固因子製剤の中間体の種類に関わらず，試験に用いた B19V（遺伝子 1 型，2 型）はいずれも動物パルボウイルスと比較して，STIM-4 蒸気加熱処理工程によって効果的に不活化された（Log 減少ファクター，3.5～4.8）。これより，蒸気加熱処理による B19V の効果的な不活化が示唆され，B19V に対する STIM-4 蒸気過熱処理を行った血液凝固因子製剤の安全性が高まると考えられた。	BYL-2008-0320

報告企業の意見	今後の対応
加熱処理によるウイルス不活化の程度は各製剤によって左右されるため，製剤毎に確認する必要があると考える。 弊社のポリグロビン N の製造に使用されているプール血漿においては，B19V に対する NAT を実施し，10E5 IU/mL 以上が確認された場合は，そのプール血漿を製造工程から除去している。感染リスクを完全に排除することはできないが，伝播の可能性は非常に低いと考える。	今後とも利用可能な B19V の検出方法の改善に関する情報収集に努める。

⑦

Inactivation of parvovirus B19 during STIM-4 vapor heat treatment of three coagulation factor concentrates

Andreas Berting, Jens Modrof, Ulrike Unger, Matthias Gessner, Andreas Klotz, Gerhard Poelsler, and Thomas R. Kreil

BACKGROUND: To enhance the viral safety margins, nanofiltration has been widely integrated into the manufacturing process of plasma-derived medicinal products. Removal of smaller agents such as parvovirus B19 (B19V) by filtration, however, is typically less efficient. Because recent investigations have demonstrated that B19V may be more heat sensitive than animal parvoviruses, the potential B19V inactivation by a proprietary vapor heating procedure (STIM-4) as incorporated into the manufacturing processes of several nanofiltered coagulation factor concentrates was investigated.

STUDY DESIGN AND METHODS: An infectivity assay based on quantitative reverse transcription–polymerase chain reaction (TaqMan, Applied Biosystems) detection of B19V mRNA after inoculation of a permissive cell line (UT7 Epo S1 cells) was used to investigate the virus inactivation capacity of the STIM-4 vapor heat treatment as used during the manufacture of nanofiltered second-generation Factor VIII inhibitor-bypassing activity (FEIBA), F IX complex, and FVII products.

RESULTS: In contrast to animal parvoviruses, both B19V genotypes investigated, that is, 1 and 2, were shown to be surprisingly effectively inactivated by the STIM-4 vapor heat treatment process, with mean log reduction factors of 3.5 to 4.8, irrespective of the product intermediate tested.

CONCLUSION: The newly demonstrated effective inactivation of B19V by vapor heating, in contrast to the earlier used animal parvoviruses, results in significant B19V safety margins for STIM-4–treated coagulation factor concentrates.

To further enhance the safety margins of plasma-derived medicinal products against any residual virus safety concerns, manufacturers have continuously sought to implement dedicated virus reduction steps into the manufacturing processes of these products. Once robustly established and widely available, nanofiltration has frequently been considered an option for this purpose (for review see Burnouf and Radosevich[1]).

In selecting the appropriate pore sizes of these filters, commercially available between 15 and 75 nm, a delicate balance needs to be struck between maintaining an appropriate yield of the respective product intermediate while effectively removing viruses. Especially for larger-molecular-weight protein preparations the removal of smaller viruses has thus been difficult,[2] unless virus antibodies present in the intermediate increased the effective filtration size of a virus by formation of virus–antibody complexes,[3,4] or specific product formulations contributed to virus removal by inducing virus aggregation.[5] Particularly parvovirus B19 (B19V), currently the only known parvovirus associated with significant pathogenicity for humans, can thus often not be efficiently removed from larger-molecular-weight biologic entities of medicinal importance by these procedures.

B19V contaminates human blood or plasma donations, at reported frequencies of 1 in 800-5950[6,7] and levels

ABBREVIATIONS: B19V = parvovirus B19; FEIBA = Factor VIII inhibitor-bypassing activity; LRF(s) = log reduction factor(s); MMV = mice minute virus; NF/VH = nanofiltered and vapor heat treated.

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TRANSFUSION ;**:**-**.

of viremia up to 10^{12} genome equivalents (geq) per mL.[6,8] Polymerase chain reaction (PCR) testing of the plasma supply has thus become state-of-the-art, and use of the technology has reduced the mean B19V load of plasma manufacturing pools by many orders of magnitude. Given the wide prevalence of B19V, however, supply considerations have prevented eliminating the virus from plasma by PCR testing, and thus virus reduction during the manufacturing process remains the critical safeguard of final product safety also in this instance.

Before an infectivity assay for B19V itself was available, animal parvoviruses, for example, porcine, murine, bovine, etc., parvoviruses, were used as so-called "model viruses"[9] in studies validating the virus reduction capacity of the manufacturing processes of plasma proteins. Based on the very high physicochemical resistance of these animal parvovirus models, virus inactivation procedures incorporated into these processes were considered less effective against parvoviruses.[10] More recently, however, initial data obtained with a novel infectivity assay for B19V itself indicated that the actual virus of concern for humans is much more heat sensitive than the animal parvoviruses used for earlier validation studies.[11-13]

Adding to the complexity, several more recently discovered human parvoviruses, for example, V9[14] and A6,[15] have now been reclassified to taxonomically represent B19V genotypes rather than distinct parvovirus species.[16]. Little is known, however, about the biologic properties of these newer B19Vs, for example, the sensitivity of these to inactivation.[17]

In this study the B19V reduction capacity of a proprietary and dedicated virus inactivation step was investigated, that is, the STIM-4 vapor heat treatment, in direct comparison to mice minute virus (MMV), an earlier used animal parvovirus model. The procedure was investigated with intermediates of several different coagulation factor concentrates that had been upgraded with respect to virus safety margins by implementation of nanofiltration during their manufacture, that is, Factor (F)VIII inhibitor-bypassing activity–nanofiltered and vapor heat treated (FEIBA NF/VH), F IX complex NF/VH, and FVII NF/VH.

MATERIALS AND METHODS

Viruses, cells, and infectivity assay

As a source of B19V, highly viremic plasma donations (990237, Genotype 1, 11.8 log geq/mL; IM81, Genotype 2, 11.4 log IU/mL) as identified by the routine plasma screening procedure of Baxter Bioscience were used. B19V were titrated on UT7 Epo S1 cells (provided by Dr Kevin E. Brown, Virus Reference Department, Center for Infections, Health Protection Agency, London, UK; with permission from Dr Kazuo Sugamura, Department of Microbiology and Immunology, Tohoku University, Graduate School of Medicine, Tohoku, Japan), essentially as earlier described.[18] Briefly, mRNA of infected cells was isolated and quantified by reverse transcription (RT)-PCR with the following procedure. Initially serial 10-fold dilutions of B19V samples of known PCR titer were incubated with UT-7 cells, and the B19V mRNA analyzed by RT-PCR. A regression line of the samples' known PCR titers versus the number of RT-PCR cycles required to obtain a positive signal for the same sample was then plotted to form a calibration curve. With this calibration curve, the PCR titer of any unknown sample was back-calculated from the mRNA RT-PCR titer obtained after incubation with susceptible cells. Typically, several 10-fold dilutions of unknown samples were analyzed by RT-PCR, to ensure that one or several of the results would lie on the linear part of the calibration curve. Whenever more than one result fitted onto the calibration curve, means were calculated for the PCR titer. The limit of detection was 3.7 log per mL,[18] and standard errors of means for multiple measurements were always not more than 0.5 log.

MMV, strain prototype (ATCC VR-1346, American Type Culture Collection, Rockville, MD) was propagated and titrated on A9 cells (ATCC CCL-1.4). Samples containing MMV were titrated by $TCID_{50}$ assay, that is, eightfold replicates of serial half-log sample dilutions were incubated with cells for 7 days before evaluation for a cytopathic effect. MMV concentrations were calculated according to the Poisson distribution and expressed as log $TCID_{50}$ per mL.

RT-PCR

For detection of B19V Genotype 1, primers sets for two mRNA splicing variants (splicing at nucleotide 1910 or 2030, Accession Number M13178[19]) were used (PA3 or PA4, respectively): PA3—primers PA3F (positions 365-386), PA3R (positions 1957-1978), and the fluorescent probe PA3P (5′-6-FAM-TTTGTGAGCTAACTAACAGATGCCCTCC ACCCAGAC-TAMRA-3′); and PA4—primers PA4F (positions 367-389), PA4R (2080-2102), and the fluorescent probe PA4P (5′-6-VIC-TGAGCTAACTAACAGGCGCCTGG AACA-TAMRA-3′).

For detection of B19V Genotype 2 (Accession Number AY903437[17]), the primer set G2 was used, G2-F (positions 369-391), G2-R (positions 1962-1983), and the fluorescent probe G2-P (5′-6-FAM-TTTGCCTGCTAATTAACAGATGCC CTCCACCCAGAC-3′).

Downscaled manufacturing processes for plasma derivatives

Downscaled versions of the manufacturing processes examined were established and the equivalence of critical product and process parameters to the respective manufacturing-scale processes established. Temperature is a critical process measure for virus inactivation and was

therefore monitored throughout all the processes investigated. Starting materials were process intermediates obtained from the manufacturing scale, which were spiked 1 in 10 with virus stock suspensions. Immediately after spiking, samples were drawn and titrated to confirm the amount of virus added. Further samples were collected and titrated at predetermined points throughout and at the end of the inactivation processes. MMV-spiked samples were directly titrated on A9 cells, whereas B19V-spiked samples were titrated on UT7 cells followed by mRNA isolation as described earlier.[18] Specific unspiked process intermediates were obtained from control procedures and tested for their potential cytotoxicity for the indicator cell line and for their potential interference with the detection of low virus titers. Virus reduction factors for the manufacturing processes examined were calculated in accordance with Committee for Proprietary Medicinal Products guidance.[9]

During their manufacture, the investigated products (all from Baxter BioScience, Zurich, Switzerland), that is, FEIBA NF, F IX complex NF (PPK NF), or together with FVII NF (prothrombin complex NF, PKT NF), are subjected to the STIM-4 vapor heating process. Specifically, a lyophilized intermediate of 7 to 8 percent residual moisture is heat treated for a minimum of 500 minutes at 60 ± 0.5°C, followed by heating to 80 ± 0.5°C, and then heating at 80 ± 0.5°C for 60 minutes. The downscaled versions of these processes were performed at the lower limits of these temperature and incubation time specifications or just below those specified for the manufacturing-scale process. To provide further assurance regarding the robustness of the virus inactivation by these processes, separate runs were performed at the upper and lower limits of the residual moisture content specified for manufacture; or runs were performed within these specifications.

Determination of the residual moisture was performed by the Karl Fischer method for non–virus-spiked control samples. The residual moisture content for all samples, including those containing virus, was confirmed by NIRVIS spectroscopy (System NIRVIS, Büchi Ltd, Flawil, Switzerland).

Product intermediates of 14.4 to 33 g per L protein concentration, 5 to 6 g per L salt concentration, and pH 7.0 to 8.0 were spiked with virus, lyophilized, and then heat-treated according to the procedure described above. Specific product measures, e.g., FEIBA (clotting assay), FII activity (clotting assay), FX activity (chromogenic assay), FVII activity (chromogenic assay), F IX activity (chromogenic assay), and protein concentration were determined for the downscale intermediate before and after the vapor heating process. The results were compared with the respective values for intermediates from the manufacturing scale to confirm equivalence of the different scale processes.

RESULTS

Vapor heating of FEIBA NF/VH

FEIBA intermediate was spiked with either B19V or MMV for downscaled vapor heating experiments. For B19V, two different primer sets (PA3, PA4) specifically designed to detect two different B19V VP1/VP2 splicing variants[20] were used for RT-PCR analysis (TaqMan, Applied Biosystems, Foster City, CA).

As can be seen in Table 1, significant inactivation of B19V was observed already after the 60°C heating phase of the process (experimentally conducted at 59.5 ± 0.5°C, i.e., worst case with respect to virus inactivation) with individual log reduction factors (LRFs) of 3.9 to 4.5. At completion of the 80°C heating phase of the process

TABLE 1. Inactivation kinetics of MMV and B19V during freeze-drying followed by vapor heating of FEIBA NF/VH process intermediates*

Percent residual moisture content:	MMV†		B19V‡					
	7	8	7-8					
Primer pairs:			Titration 1		Titration 2		Titration 3	
			PA3	PA4	PA3	PA4	PA3	PA4
Virus stock suspension	8.3	8.2	11.2	11.2	11.8	11.8	ND	ND
Spiked process intermediate§	7.2	7.2	10.3	10.4	10.6	10.4	10.6	10.4
Spiked and lyophilized intermediate	6.6	6.7	9.7	9.9	10.0	9.9	9.8	9.8
Heated at 59.5°C ± 0.5°C, 180 ± 5 min	ND	ND	7.8	7.7	7.7	7.6	ND	ND
Heated at 59.5°C ± 0.5°C, 505 ± 5 min	6.7	6.7	6.2	5.9	6.6	6.5	6.5	6.5
Reduction factor (after 60°C phase)	0.5	0.5	4.1	4.5	4.0	3.9	4.1	3.9
Heated at 79.5°C ± 0.5°C, 55 ± 5 min§	6.3	6.3	5.7	5.6	5.5	5.4	5.7	5.8
Reduction factor	0.9	0.9	4.6	4.8	5.1	5.0	4.9	4.6
Mean reduction factor	0.9				4.8			

* For the detection of spliced B19V mRNA two different primer sets, i.e., PA3 and PA4, were used.
† MMV titers are expressed as [log($TCID_{50}$/mL)].
‡ B19V titers are expressed as [log geq/mL].
§ Titers at this sampling stage were used to calculate the virus reduction factor after the entire heating phases.
ND = not determined.

(experimentally conducted at 79.5 ± 0.5°C), some residual B19V infectivity was still detectable, although LRFs of 4.6 to 5.1 were obtained, with a mean LRF of 4.8. Because use of primer sets PA3 and PA4 resulted in fully equivalent results, only one of the primer sets (PA3) was used for the detection of B19V Genotype 1 mRNA in further experiments.

In contrast to the effective inactivation of B19V by the vapor heating process, the inactivation observed for the animal parvovirus MMV was insignificant, with a mean LRF of 0.9.

Vapor heating of F IX complex NF/VH

To investigate the vapor heating process of F IX complex, the respective intermediate was spiked with either B19V or MMV. Because residual moisture during the vapor heating process might be considered a critical parameter for the effectiveness of virus inactivation, separate vapor heating runs were performed at the upper and lower limit of the specified residual moisture content of the manufacturing process, that is, 7 and 8 percent (wt/wt), to investigate the robustness of virus inactivation by the process. For B19V-spiked runs, some residual infectivity was still detected after the entire heating process, but the results obtained demonstrate comparable reduction factors for runs at either 7 or 8 percent residual moisture content with a mean LRF of 4.6 (Table 2). A substantial inactivation of B19V was observed already after the 60°C heating phase of the process (investigated at 59.5 ± 0.5°C) with individual LRFs of 3.7 to 4.2. As the reduction factors obtained between the individual titrations at 7 and 8 percent residual moisture content were comparable, consequently, the following vapor heating experiments were performed at between 7 and 8 percent residual moisture content, that is, within the specifications of the large-scale process.

MMV, again in sharp contrast to the effective inactivation of B19V by the vapor heating process, was not significantly inactivated even at the end of the entire heating process, with a mean LRF of 0.9. As seen with B19V before, there were again no differences between MMV inactivation results for individual heating runs conducted at 7 and 8 percent residual moisture.

Vapor heating of FVII NF/VH

F IX complex and FVII are separately produced and are individual products. Both components can, however, also be combined to the prothrombin complex total product. Because FVII is the second component of prothrombin complex total, the B19V and MMV inactivation by STIM-4 vapor heating was also investigated.

At the end of the entire heating phase, effective inactivation of B19V was observed, with a mean LRF of greater than 4.0 (Table 3). Also, substantial inactivation of B19V was found already after the 60°C heating phase (investigated at 59.5 ± 0.5°C) of the process (LRFs of 4.0 and 4.5), confirming earlier findings for the other prothrombin complex total compound. Again in sharp contrast to effective B19V inactivation, the parvovirus model MMV was inactivated only ineffectively, with a mean LRF of 1.7.

STIM-4 inactivation of B19V Genotype 1 versus Genotype 2: FEIBA, for example

To understand any potentially different thermosensitivity of the recently classified B19V Genotype 2, versus the earlier investigated B19V Genotype 1, FEIBA intermediate was now spiked with B19V Genotype 2 and treated as described earlier (see "Vapor heating of FEIBA NF/VH"). mRNAs isolated after culture with UT-7 cells were analyzed by TaqMan RT-PCR with either, as before, Genotype 1 primer sets (PA3), or now also specific Genotype 2 (G2) primer sets.

TABLE 2. Inactivation kinetics of MMV and B19V during freeze-drying followed by vapor heating of F IX complex NF/VH intermediate

	MMV*		B19V†			
Percent residual moisture content:	7	8	7		8	
			Titration 1	Titration 2	Titration 1	Titration 2
Virus stock suspension:	7.4	7.5	ND	ND	ND	ND
Spiked process intermediate‡	6.5	6.7	10.7	10.6	11.0	11.0
Spiked and lyophilized intermediate	6.5	6.4	10.8	10.5	10.1	10.4
Heated at 59.5°C ± 0.5°C, 495 ± 5 min	6.1	6.2	6.9	6.9	7.1	6.8
Reduction factor (after 60°C phase)	0.4	0.5	3.8	3.7	3.9	4.2
Heated at 79.5°C ± 0.5°C, 55 ± 5 min‡	5.8	5.6	6.4	6.6	5.8	6.3
Reduction factor	0.7	1.0	4.3	4.1	5.2	4.7
Mean reduction factor (log)	0.9		4.6			

* MMV titers are expressed as [log($TCID_{50}$/mL)].
† B19V titers are expressed as [log geq/mL].
‡ Titers at this sampling stage were used to calculate the virus reduction factor after the entire heating phases.
ND = not determined.

TABLE 3. Inactivation kinetics of MMV and B19V during freeze-drying followed by vapor heating of FVII NF/VH intermediate

Percent residual moisture content:	MMV*		B19V†			
	7	8	7-8			
			Run 1		Run 2	
			Titration 1	Titration 2	Titration 1	Titration 2
Virus stock suspension:	7.7	8.1	12.3	ND	ND	ND
Spiked process intermediate‡	6.5	7.2	11.0	10.6	10.8	10.8
Spiked and lyophilized intermediate	6.9	7.0	ND	9.9	9.9	10.1
Heated at 59.5°C ± 0.5°C, 525 ± 5 min	5.3	6.1	6.5	6.6	positive§	positive§
Reduction factor (after 60°C phase)	1.2	1.1	4.5	4.0	ND	ND
Heated at 79.5°C ± 0.5°C, 55 ± 5 min‡	5.2	5.2	<6.8	<6.8	<6.8	<6.8
Reduction factor	1.3	2.0	>4.1	>3.8	>4.0	>4.0
Mean reduction factor	1.7		> 4.0			

* MMV titers are expressed as [log($TCID_{50}$/mL)].
† B19V titers are expressed as [log genome equivalents/mL].
‡ Titers at this sampling stage were used to calculate the virus reduction factor.
§ These samples were tested positive; a titer could, however, not be calculated, because the PCR cycle numbers necessary to obtain a positive fluorescence signal were outside the range covered by the mean regression line.
ND = not determined.

TABLE 4. B19V Genotype 2 inactivation by vapor heat treatment during the manufacture of FEIBA NF/VH*

Percent residual moisture content:	B19V GT 2			
	7-8			
	Run 1	Run 2	Run 1	Run 2
	PA3	PA3	G2	G2
Reduction factor†	>3	4	>3	>4
Mean reduction factor	3.5			

* The TaqMan RT-PCR was performed with either Genotype 1 (PA3)- or Genotype 2 (G2)-specific primer sets.
† Reduction factor determined after the entire vapor heating procedure. The goodness-of-fit values of the standard regression lines were less than optimal resulting in a high standard deviation at lower virus titers. Therefore, reduction factors were determined by the difference in integer log sample dilutions between the spiked intermediate and the sample after completion of vapor heating.

As can be seen, use of both the two primer sets (PA3/PA4) designed for two Genotype 1 splicing variants revealed highly comparable results (Table 1). In addition insignificant differences between B19V Genotype 1 and Genotype 2 occurred, with both viruses effectively inactivated by the STIM-4 vapor heating process (Tables 1 and 4). Specifically, here for B19V Genotype 2, effective inactivation was observed at the end of the heating process, with calculated LRFs between greater than 3 and greater than 4, that is, a mean LRF of 3.5. Using the Genotype 1 (PA3)- or the Genotype 2 (G2)-specific primer sets, highly comparable inactivation results were obtained.

DISCUSSION

Since its discovery in 1975,[21] B19V has been associated with an ever-broadening panel of diseases. While initially only known as the causative agent of an erythematous childhood disease (fifth disease), more recently the virus is appreciated as the causative agent of more severe diseases such as, for example, hydrops fetalis,[22] arthritis,[23] hepatitis,[22] and possibly a significant number of myocarditis cases.[24]

The introduction of B19V PCR testing of plasma for fractionation, as initially defined under the Plasma Protein Therapeutics Association's voluntary standards,[25] has reduced plasma pool loads of the virus by several orders of magnitude[26] and correspondingly enhanced the B19V safety margins of plasma products. In support of the notion, while episodes of B19V transmissions have historically occurred,[27] such reports have not been received for the implicated products after the introduction of B19V PCR testing. There is, however, still a residual concern around the potential B19V contamination of plasma.

The final safeguard of product safety, that is, the virus reduction that occurs during the manufacturing process, has thus been of particular interest, also with respect to B19V. Unfortunately though, the lack of a widely available B19V infectivity assay has forced studies aimed at validating the B19V reduction capacity of manufacturing processes to be conducted with animal parvoviruses as "model viruses." Where these viruses are particularly resistant to physicochemical inactivation, the results obtained were often less reassuring.

There was consequently significant interest in a suitable B19V assay to investigate the virus of concern itself, and development efforts were lately rewarded. Initial use of the newly available approaches revealed that B19V itself was significantly more susceptible to inactivation by, for example, pasteurization,[11] low pH,[28] and dry heat,[13] than the earlier used animal parvovirus models. Also, however, research conducted with these assays has indicated that B19V has unique properties in terms of heat sensitivity

and its inactivation is particularly dependent on the composition of the matrices during (liquid) heating.[29]

The proprietary STIM-4 vapor heating process is a heat treatment step conducted at lyophilized product of 7 to 8 percent residual moisture that has been incorporated in the manufacturing process of several coagulation factor concentrates, in addition to a 35-nm nanofiltration step for FEIBA NF/VH, F IX complex NF/VH, and FVII NF/VH. Here we describe the efficient inactivation of B19V, in marked contrast to an animal parvovirus model, that is, MMV, by this vapor heat treatment.

The results of the two-phase vapor heat treatment demonstrate that B19V is effectively inactivated by this process step, whereas MMV is only marginally reduced. By use of both the two primer sets (PA3/PA4) designed to detect two Genotype 1 VP1/VP2 splicing variants highly comparable results were obtained (Table 1), indicating that the splicing variants in infected UT7-Epo S1 cells occur in rather similar concentrations.

After the entire heating process for B19V Genotype 1 mean log reduction factors of 4.8, 4.6, and more than 4.0 were obtained with highly comparable results for the panel of coagulation factor intermediates investigated, that is, FEIBA, F IX complex and FVII (see Tables 1-3). These findings support the robustness of the STIM-4 vapor heat treatment in inactivating B19V. Moreover, the significant inactivation of B19V already after the first heating phase at 60°C for the coagulation factors investigated (see Tables 1-3) provides further reassurance. In addition, varying the residual moisture content during the heat treatment, that is, to the lower (7%) and the upper limit (8%) specified for the manufacturing process, still resulted in highly comparable inactivation of B19V (Table 2).

The discovery of additional human erythrovirus genotypes, that is, Genotypes 1, 2, and 3,[16] and also novel parvoviruses,[30,31] has raised new questions about their biologic properties, their pathogenic potential and also their relevance to the viral safety of plasma-derived products. B19V Genotype 2 has been detected in human blood at high titers, and recently this genotype has also been found in a few lots of plasma-derived coagulation factor concentrates; all of them, however, were cocontaminated with Genotype 1 DNA.[32] These findings indicate that Genotype 2 has established a moderate prevalence in the population and therefore investigations regarding the inactivation of this B19V genotype may also be desirable. Recent studies that investigated the inactivation capacity of liquid heating and low pH[17] incubation for B19V Genotypes 1 and 2 in parallel suggested comparable inactivation capacity and kinetics and thus maybe also comparable physicochemical properties for the virus particles of both genotypes.[17]

In this report, the physicochemical stability of virus particles of Genotype 1 and 2 were compared during the vapor heat treatment process step described. The results demonstrate that both B19V genotypes are inactivated with comparable mean LRFs of 4.8 and 3.5 (Tables 1 and 4). These data indicate that B19V Genotype 1 and 2 particles have very similar physicochemical properties and thus data obtained in studies with Genotype 1 should also be indicative for Genotype 2 behavior. This argument is strengthened by very recent findings that anti-B19V–positive plasma samples or intravenous immune globulin product were able to neutralize B19V Genotype 1 and Genotype 2.[18] Furthermore, studies by Ekman and colleagues[33] suggest that all three B19V genotypes are similar variants of the same species and constitute a single serotype. Not surprising in this context, the pathogenic potential of different B19V genotypes also seems to be comparable.[14]

Altogether the results of this study demonstrate that the STIM-4 vapor heat treatment is a highly effective and robust virus inactivation step for the relevant parvovirus B19, both Genotype 1 and Genotype 2. Specifically, the STIM-4 vapor heat treatment process substantially contributes to the safety margins of the plasma-derived products FEIBA NF/VH, F IX complex NF/VH, and FVII NF/VH. As suggested by a recent article,[29] however, the inactivation capacity of heat treatment may significantly depend on the specific matrix investigated, and thus B19V inactivation needs to be confirmed for every specific product and process individually.

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