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*

	MMV†		B19V‡					
Percent residual moisture content:	7	8	7-8					
			Titration 1		Titration 2		Titration 3	
Primer pairs:			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

	MMV*		B19V†			
Percent residual moisture content:	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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