

Biosystems) with a RT-PCR core reagent kit (TaqMan EZ, PE Applied Biosystems). The B19V mRNA primers amplify a region from nucleotide position nucleotide 365 to nucleotide 1978, spanning splice donor site nucleotide 406 to splice acceptor site nucleotide 1910 (reference sequence PVBAUA, NCBI GeneBank, Accession Number M13178), that is, effectively preventing B19V DNA amplification by use of an intron-spanning probe. As control for mRNA quality and to exclude false-negative B19V results, all samples were tested in parallel for the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a predeveloped TaqMan assay reagents human GAPDH kit (PE Applied Biosystems).

Amplification of B19V mRNA was performed with 640 nmol per L each primer (PA3F, 5'-TTCTCTGGAC TTTCTTGCTGTT-3'; PA3R, 5'-CACCACTGCTGCTGA TACT-3'), 160 nmol per L the intron-spanning probe (PA3P, 5'-VIC-TTTGTGAGCTAACTAACAGATGCCCTCCAC CCAGAC-TAMRA-3'), and 10 ng of mRNA. Appropriate reagent controls and 200 copies of in vitro transcribed control RNA (10 copies/ $\mu$ L) in duplicate were included on each microtiter plate. Primers and probe for B19V PCR (synthesized by Ingenetix, Vienna, Austria) were high-performance liquid chromatography purified to a purity of greater than 90 percent.

The amplification program for GAPDH and B19V was composed of reverse transcription of mRNA into cDNA (50°C for 2 min and 60°C for 20 min) and subsequent PCR amplification with an initial denaturation step at 95°C for 5 minutes and 45 cycles of denaturation at 94°C for 20 seconds and annealing and extension at 57°C for 1 minute. Analysis of data generated was performed with the TaqMan software (SDS software Version 2.0, PE Applied Biosystems) with a threshold of 0.07  $\Delta$ Rn fluorescent signal (label, VIC).

#### B19V neutralization with plasma pools or IVIG

To investigate B19V neutralization at B19V IgG titers of 11 IU per mL or below, two single plasma donations that had been tested nonreactive for B19V IgG were blended with plasma from manufacturing pools (cryorich plasma) to obtain defined titers between 0.4 and 11 IU per mL B19V IgG. The actual B19V IgG titers of these plasma pool blends (4 and 11 IU/mL) were confirmed by ELISA (Novagnost Parvovirus B19 IgG-ELISA; NovaTec, Dietzenbach, Germany).

A quantity of 450  $\mu$ L of plasma pool blends or original plasma pool samples (cryorich plasma) were mixed with 50  $\mu$ L of cell culture medium containing between 10<sup>2</sup> and 10<sup>10</sup> IU B19V. After incubation for 1 hour at 37°C, the entire 500- $\mu$ L mixture was incubated on 10<sup>5</sup> UT7/Epo-S1 cells for 7 days, before testing B19V infectivity by RT-PCR as described above. To control for any potential complement influence, neutralization experiments were also per-

formed after incubation of B19V IgG containing plasma samples at 56°C for 30 minutes before use. As a control, B19V titers between 10<sup>2</sup> and 10<sup>10</sup> IU were incubated with the two single plasma donations (both tested nonreactive for B19V IgG and B19V) instead of the B19V IgG containing plasma pool blends or original plasma pools for each experiment.

For each B19V IgG concentration used for neutralization tests at least four (0.4, 4, and 11 IU/mL) or two (1 and 36 IU/mL) independent titrations were performed. A human IVIG product (for Europe, KIOVIG [Baxter Healthcare Corporation, Westlake Village, CA]; for the United States, Gammagard liquid [Baxter Healthcare Corporation]) was used in neutralization tests as described. Because the original IVIG preparation had a B19V IgG titer of 562 IU per mL (manufactured from plasma pools with a mean B19V IgG titer of 40 IU/mL), dilutions ranging in B19V IgG titer from 0.1 to 25 IU were prepared in 0.2 mol per L glycine, the medium of the IVIG preparation.

The results obtained by duplicate TaqMan RT-PCR runs for each sample were analyzed qualitatively, that is, crossing of the TaqMan RT-PCR threshold of 0.07  $\Delta$ Rn VIC was scored positive while not exceeding that background threshold was scored negative.

#### Statistical analysis

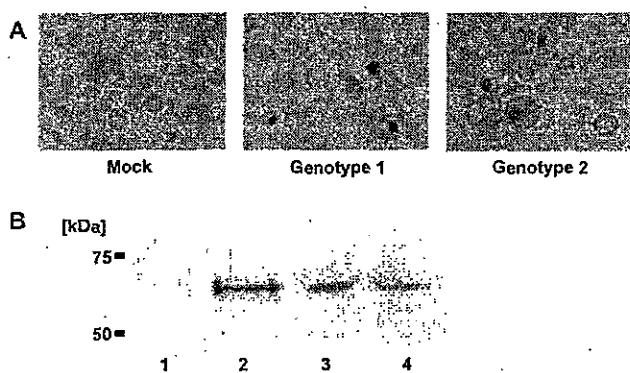
Statistical evaluation of B19V IgG titer frequencies in manufacturing plasma pools (containing a few thousand donations each), the calculation of B19V titers that equaled 50 percent infectivity and unpaired t tests were calculated with computer software (GraphPad Prism 4, GraphPad Software, San Diego, CA).

## RESULTS

#### Infection of UT7/Epo-S1 cells with B19V Genotype 1 and 2

Although it had been shown that B19V Genotype 1 infectivity can be quantified by RT-PCR analysis of mRNA isolated from infected UT7/Epo-S1 cells, it was suggested that this same assay might not work for B19V Genotype 2.<sup>22</sup> To reinvestigate the susceptibility of UT7/Epo-S1 cells for infection with B19V Genotype 2, cells were in parallel exposed to either B19V Genotype 1 or B19V Genotype 2, and the following events were examined by immunocytochemical staining and Western blot (Fig. 1).

Immunocytochemistry of cells prepared on Day 1 after B19V infection served as negative control that confirmed that inoculum virus was sufficiently removed. Cells prepared on Day 3 after infection, however, revealed the presence of newly synthesized B19V capsid proteins within infected UT7/Epo-S1 cells, after exposure to both B19V Genotype 1 or B19V Genotype 2 (Fig. 1A). As sug-



**Fig. 1. Infection of UT7/Epo-S1 cells with B19V Genotypes 1 and 2.** (A) Immunocytochemical staining of UT7/Epo-S1 cells that were mock-infected, infected with B19V Genotype 1 (MOI, 10<sup>6</sup>) or B19V Genotype 2 (MOI, 10<sup>5.6</sup>) for 7 days. Original magnification,  $\times 40$ . (B) Western blot of cell lysates from UT7/Epo-S1 cells. Lane 1 = negative control, mock-infected cells; Lanes 2 through 4 = B19V-infected cells; Lane 2 = Genotype 1 (MOI, 10<sup>6</sup>); Lane 3 = Genotype 1 (MOI, 10<sup>6</sup>); Lane 4 = Genotype 2 (MOI, 10<sup>5</sup>).

gested by Fig. 1A, and confirmed by counterstaining of infected cells with methyl green (not shown); the VP1/VP2 staining was primarily confined to the nucleus. Quantitatively, approximately 10 percent cells infected with either Genotype 1 or Genotype 2 were positive on Day 3 after infection. Seven days after infection UT7/Epo-S1 cells had multiplied to an approximately 10-fold higher cell number, yet the percentage of infected cells was still at approximately 10 percent (data not shown).

The detection of B19V capsid proteins by Western blot also confirmed the susceptibility of UT7/Epo-S1 cells to infection with both B19V Genotype 1 and B19V Genotype 2 (Fig. 1B). The predominant B19V capsid protein splice variant VP2 was clearly detectable for both B19V genotypes at 7 days postinfection, although the signal for Genotype 1 appeared somewhat stronger compared to the signal for Genotype 2. This effect became more apparent when the MOI used for Genotype 1 was 10 times lower than that for Genotype 2 (Fig. 1B, Lanes 3 and 4). Cell homogenates obtained during Days 1 through 4 after infection did not result in detectable Western blot signals, confirming de novo synthesis of B19V proteins rather than detection of residual inoculum virus particles. The detection limits of B19V infectivity determined by immunocytochemical staining and Western blot were for both approximately 10<sup>3</sup> IU B19V.

The presence of B19V proteins in infected UT7/Epo-S1 cells as now demonstrated for both B19V Genotype 1 and B19V Genotype 2 would, as a prerequisite, require production of B19V-specific mRNAs, spliced exactly as during infection of humans.<sup>24</sup> The presence of these spliced mRNA species would then provide the basis

for detection of B19V infectivity by TaqMan RT-PCR as described under Material and Methods.

As expected, RT-PCR confirmed infectivity of B19V Genotypes 1 and 2 for UT7/Epo-S1 cells. For an accurate calculation of the B19V PCR titer that corresponds to one 50 percent tissue culture-infectious dose (TCID<sub>50</sub>), at least eight replicates of the B19V titers that did not result in all negative or in all positive RT-PCR results were analyzed (Table 1). The respective TCID<sub>50</sub> values obtained were 3.7 log IU for B19V Genotype 1 versus 6.1 log IU for B19V Genotype 2.

### Neutralization of B19V

Plasma manufacturing pools consist of typically several thousand individual donations and at a B19V IgG seroprevalence of approximately 30 to 60 percent in the plasma-donating population<sup>10</sup> statistically rather evenly distributed B19V antibody levels should be expected to occur in plasma pools. To establish a statistically meaningful estimate of the B19V antibody concentrations present in plasma manufacturing pools, samples were obtained from a total of 1174 pools, representing a few million donations collected in either Europe or the United States over the course of 2 years, to be tested for B19V antibodies by ELISA.

As expected from the high seroprevalence of B19V in the human population, the analysis revealed a rather high mean ELISA titer of 33  $\pm$  9 IU per mL (mean  $\pm$  SD) among all the plasma pools tested, with a high of 71 IU per mL and a low of 11 IU per mL (Fig. 2A). Although thus the presence of a varying yet significant level of B19V antibodies had been confirmed for a statistically relevant number of plasma pools by ELISA, we sought to measure antibody function—rather than presence—considering this clinically more meaningful.

Neutralization of B19V infectivity in plasma pool specimens was thus investigated, utilizing the detection of B19V infectivity by TaqMan RT-PCR, after incubation in the presence or absence of defined concentrations of B19V IgG as determined by ELISA. Of potentially the most significant clinical importance, the B19V neutralization capacity at the lowest ELISA titer ever found for a plasma manufacturing pool in our investigation, that is, 11 IU per mL, was investigated (Fig. 2A). The test material was derived by blending plasma manufacturing pool specimens of higher B19V antibody titer (36–40 IU/mL B19V IgG) with plasma donations individually screened to be nonreactive for B19V IgG and B19V, to reach the target concentration of 11 IU per mL B19V IgG.

The comparison of B19V Genotype 1 infectivity after incubation with either B19V antibody-negative plasma (control) or the plasma pool sample blended to contain 11 IU per mL B19V IgG can be seen in Fig. 2B. For each data point given in Fig. 2B, at least five infectivity tests

TABLE 1. Infectivity of B19V Genotypes 1 and 2, detected by mRNA TaqMan RT-PCR\*

B19V (IU) for infection	Detection of B19V infectivity by mRNA RT-PCR	
	Genotype 1	Genotype 2
10 <sup>10</sup>	2+	8+
10 <sup>9</sup>	2+	12+
10 <sup>8</sup>	2+	7+/-
10 <sup>7</sup>	2+	10+/-
10 <sup>6</sup>	18+	5+/-
10 <sup>5</sup>	12+	6-
10 <sup>4</sup>	9+/-	NT
10 <sup>3</sup>	1+/-	NT
10 <sup>2</sup>	10-	NT
TCID <sub>50</sub> (log)	3.7	6.1

\* Seven days after infection of UT7/Epo-S1 cells with different B19V concentrations, mRNA was isolated and subjected to TaqMan RT-PCR. From the numbers of positive (+) and negative (-) results obtained at certain virus dilutions, the virus titer corresponding to 50 percent infectivity (TCID<sub>50</sub>) was calculated.

NT = not tested.

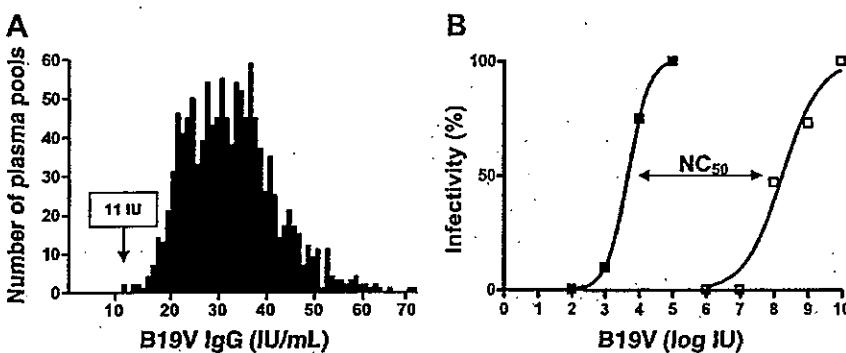


Fig. 2. Neutralization of B19V by plasma manufacturing pools. (A) B19V IgG antibody titers of 1174 plasma manufacturing pools as determined by ELISA (IU/mL). (B) Infectivity (% detected by TaqMan mRNA RT-PCR) of B19V samples on UT7/Epo-S1 cells that were inoculated with 10<sup>2</sup> to 10<sup>9</sup> IU B19V (■) or 10<sup>6</sup> to 10<sup>10</sup> IU B19V (□). The difference in B19V concentrations that corresponds to 50 percent B19V infectivity between the two sets of samples represents the NC<sub>50</sub> of B19V IgG at 11 IU per mL.

have been performed to determine the percentage of infectivity from the respective number of positive and negative TaqMan RT-PCR results. Compared to the TCID<sub>50</sub> of 3.7 log IU per mL for B19V Genotype 1 in the presence of control plasma, incubation with the plasma containing 11 IU per mL B19V IgG increased the TCID<sub>50</sub> to a corresponding B19V concentration of 8.2 log IU per mL. From these results, the 50 percent B19V neutralization capacity (NC<sub>50</sub>) for plasma containing 11 IU per mL B19V IgG could be calculated, that is, the difference of both 50 percent infectivity calculations, as 4.6 log B19V IU per mL.

To substantiate the relevance of this result that was obtained by use of blended plasma specimens, samples from the two original plasma manufacturing pools from 2004 and 2005, each containing only 11 IU B19V IgG per mL, were also investigated in the same neutralization

assay setup. Although the available sample volumes from these two specific plasma manufacturing pools were limited, only three neutralization assays could be performed with the original pool samples, demonstrating a mean NC<sub>50</sub> neutralization capacity of 4.6 log B19V IU per mL, thus confirming the results earlier obtained with the blended plasma samples.

To establish a dose-response relationship between the B19V neutralization capacity and the presence of B19V antibodies as determined by ELISA, samples at B19V IgG concentrations higher than the 11 IU per mL or even lower, that is, concentrations that never occur in plasma manufacturing pools, were generated with the blending approach described. These samples, at B19V antibody concentrations between 0.4 and 36 IU per mL B19V IgG, were subsequently tested for their B19V neutralization capacity (Fig. 3).

The NC<sub>50</sub> obtained for a plasma sample containing an artificially low 0.4 IU per mL B19V antibody titer was 1.8 log B19V. At 1 and 4 IU per mL B19V IgG, that is, still well below the lowest B19V IgG concentrations ever observed in a plasma manufacturing pool, 3.1 log and 4.4 log B19V IU per mL were neutralized, respectively. At B19V antibody concentrations higher than the earlier tested 11 IU, the demonstrable B19V neutralization did just marginally increase, showing a NC<sub>50</sub> of 4.9 log B19V at 36 IU per mL B19V IgG.

Neutralization experiments with B19V Genotype 2 were somehow limited by the significantly higher limit of detection for Genotype 2 compared to Genotype 1 (Table 1). Specifically, already at 11 IU per mL B19V IgG the NC<sub>50</sub> was beyond the limit of detection for the method, and the corresponding result was neutralization of greater than 3.9 log for B19V Genotype 2. This level of neutralization, however, was well comparable to the one earlier observed for B19V Genotype 1.

Because IVIG products are indicated for the treatment of severe B19V infections, information about the functional B19V neutralization capacity of such products would be clinically relevant. Consequently, the neutralization capacity of IVIG samples was tested. Because the original IVIG preparation contained 562 IU per mL B19V IgG (corresponding to 5.62 IU B19V IgG/mg IgG), predilution to contain B19V IgG titers between 0.1 and 25 IU was

required for evaluation by the neutralization assay. Coherent with the results earlier obtained for plasma samples, 2.2 log infectious B19V were already neutralized by an IVIG sample at 0.3 IU B19V antibody, and 5.2 log infectious B19V were neutralized by an IVIG specimen diluted to 8.4 IU B19V IgG.

#### Anti B19V titers in different manufacturing pools

Comparing the B19V IgG content of different US plasma manufacturing pools (pooled between January 5, 2004, to November 18, 2005), prepared from either source plasma, that is, collected by plasmapheresis, or recovered plasma, that is, derived from whole-blood donations, we found significantly different B19V IgG titers depending on the plasma source: while recovered plasma pools ( $n = 48$ ) contained a mean  $\pm$  SEM titer of  $43 \pm 1.5$  IU per mL (range 22–71 IU/mL), source plasma pools ( $n = 630$ ) only had  $31 \pm 0.3$  IU per mL (range, 11–53 IU/mL). These differences were significant, as shown by analysis of the B19V antibody titers between source and recovered plasma pools by unpaired *t* test ( $p < 0.0001$ ; Fig. 4A).

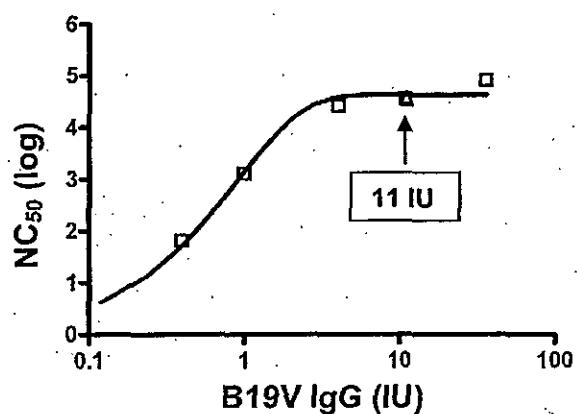


Fig. 3. Dose-response relation for neutralization of B19V. Relation of B19V infectivity  $NC_{50}$  (see Fig. 2) and concentrations of B19V IgG (IU).

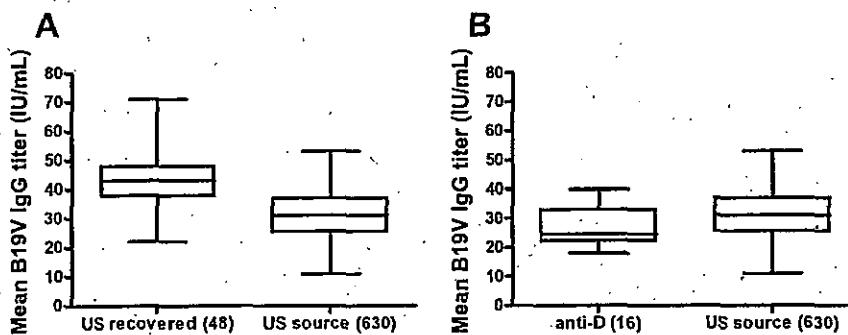


Fig. 4. Comparison of mean B19V IgG titers in plasma manufacturing pools, produced in 2004 and 2005, by unpaired *t* test. (A) US source ( $n = 630$ ) versus US recovered ( $n = 48$ ),  $p < 0.0001$ . (B) Anti-D ( $n = 16$ ) versus US source ( $n = 630$ ),  $p = 0.0694$ .

Whereas for the plasma pools so far tested for B19V antibodies an industry voluntary standard<sup>25</sup> required limiting the presence of B19V by PCR pretesting to less than  $10^5$  IU per mL, a limit of less than  $10^4$  IU per mL B19V has been mandatory for pools of anti-D plasma.<sup>26</sup> Reaching this lower limit requires interdiction of additional donations that contain B19V. As such donations might be expected to also contain B19V antibodies,<sup>6</sup> it is conceivable that the different PCR testing strategies applied for regular versus anti-D plasma might also result in somewhat different B19V IgG concentrations.

Comparing the ELISA B19V IgG results, however, for anti-D plasma ( $n = 16$ ) and for regular US source plasma ( $n = 630$ ), that is, plasma types that with respect to B19V only differ in the respective cutoff limits for PCR testing, they were not significantly different ( $p = 0.0694$ ; Fig. 4B).

#### DISCUSSION

With the emergence of the A6 and V9 erythroviruses,<sup>22,27</sup> that is, viruses that have now been reclassified as B19V genotypes, a useful assay for quantification of B19V infectivity should also be capable of quantifying these genotypes or at least Genotype 1 as the by far most prevalent and Genotype 2 that has been shown to—more rarely—also occur in plasma for fractionation. In contrast to an earlier suggestion that UT7/Epo-S1 cells might not be susceptible to B19V Genotype 2,<sup>22</sup> infection of UT7/Epo-S1 cells by both B19V Genotype 1 and Genotype 2 is demonstrated in the current work. This apparent discrepancy with earlier results is likely due to human immunodeficiency virus coinfection of the earlier used Genotype 2 B19V specimen and its heat inactivation before use in the B19V study, a procedure that possibly would inactivate B19V infectivity.<sup>18</sup> Even in our study, however, the detection of B19V Genotype 2 by Western blot was approximately 10 times less sensitive compared to Genotype 1, and with the TaqMan RT-PCR B19V the limit of detection for Genotype 1 was approximately 100 times lower than for Genotype 2 (Table 1). For the RT-PCR assay, this difference was initially considered to be the consequence of a few mismatches of the primers used for B19V Genotype 2. Another set of primers was therefore designed and tested, specifically designed for the sequence of the B19V Genotype 2 sample used.<sup>20</sup> Even with those, however, the same detection limit was determined as with the Genotype 1 specific primers, suggesting that B19V Genotype 2 infects UT7/Epo-S1 cells somewhat less efficiently compared to B19V Genotype 1, a possible explana-

tion also for the Western blot discrepancy between the two genotypes. Genotype 1 B19V was detected with one TCID<sub>50</sub> corresponding to 3.7 log IU, that is, well in agreement with results from other investigations.<sup>15,16,28</sup>

Owing to the high prevalence of B19V in the population, the titer of anti-B19V in plasma pools for manufacturing was anticipated to be high and rather constant.<sup>11</sup> Data on B19V IgG in plasma pools available so far, however, were rather limited, with only 20 to 66 plasma pools investigated with mean ( $\pm$  SD) B19V IgG contents ranging from 29.8  $\pm$  17.2 to 64.7  $\pm$  17.5 IU per mL.<sup>11-14</sup> Here a total of 1174 plasma pools were analyzed, representing a few million donations collected over a period of 2 years in both the United States and the European Union, which revealed a mean ( $\pm$  SD) B19V IgG titer of 33  $\pm$  9 IU per mL. Of all these pools, the minimum B19V IgG titer ever found was 11 IU per mL. In the following series of neutralization experiments, correlating the presence of B19V IgG (ELISA) to functional capacity and/or neutralization, B19V Genotype 1 neutralization by B19V IgG was found to be dose-dependent with a neutralization capacity greater than 4 log B19V already at only 4 IU per mL, that is, an artificially low antibody concentration compared to the naturally occurring minimal concentration in plasma pools (11 IU/mL B19V IgG). A neutralization capacity of greater than 4 log has also been shown for higher B19V IgG concentrations; at up to 36 IU per mL. Neutralization of B19V Genotype 2 with 11 IU per mL B19V IgG was likewise shown to be greater than 3.9 log, although a more exact quantification was limited by the higher limit of detection for B19V Genotype 2 in the assay used. In agreement with this, other neutralization experiments with the KU812Ep6 cell line also showed neutralization of more than 4 log B19V infectivity by less than 3.5 IU per mL B19V IgG.<sup>20</sup> In contrast to our study, however, only single plasma donations instead of plasma manufacturing pools were investigated. Even for these individual samples, Blumel and coworkers<sup>20</sup> have indicated B19V antibody cross-reactivity for different B19V genotypes. Based on the contribution of many thousand donors for any plasma manufacturing pool, it can be expected that an even broader range of different B19V genotypes can be neutralized with B19V IgG from plasma pools, a perspective of particular importance for the B19V neutralization capacity of IVIG products produced from these pools. An experimental comparison of the neutralization capacity of plasma manufacturing pools and IVIG showed that with only 8 IU B19V IgG contained within an IVIG sample greater than 5 log B19V were neutralized. A similar degree of B19V neutralization can also be extracted from recent work by another group.<sup>29</sup>

Although several reports have described the transmission of B19V through plasma derived products, the amount of B19V that represents an infectious dose in humans is not yet clear. In one anecdotal B19V transmis-

sion episode, however, solvent/detergent-treated plasma that contained 10<sup>7.5</sup> genome equivalents (geq) B19V per mL in the presence of at least 8.8 IU per mL B19V IgG transmitted B19V, while no transmission occurred at a B19V load of 10<sup>3.5</sup> geq per mL or less.<sup>30,31</sup> This observation is in good agreement with our neutralization experiments that have shown neutralization of more than 10<sup>4</sup> IU per mL B19V by even only 4 IU per mL B19V IgG (Fig. 3; note that according to an internal validation, for the B19V Genotype 1-positive donation used in this study, virus quantification in IU is equivalent to geq).

The even higher B19V neutralization capacity as shown for an IVIG product compared to plasma pools would also support the use of IVIG as treatment for fulminant B19V infections, based on the potent neutralization efficacy.<sup>9</sup> To extrapolate our data from in vitro neutralization of B19V by IVIG to the clinical situation, we made a comparison with the amount routinely administered during common variable immune deficiency treatment, that is, 100 to 400 mg immunoglobulins per kilogram of human body weight. With a typical volume of 84.4 mL blood per kilogram of body weight,<sup>32</sup> the IVIG preparation (containing immunoglobulins at 10%) is diluted 1:84 to 1:21 during common variable immune deficiency treatment. Because the IVIG preparation investigated in this study contained 562 IU per mL B19V IgG, the concentration of B19V IgG in the human blood after administration would be 7 to 27 IU per mL. We have shown in vitro neutralization of more than 5 log infective B19V with 8 IU anti-B19V IgG, thus indeed indicating efficient protection from infection with B19V conferred by IVIG.

Evidence exists to suggest that the prevalence of B19V IgG in the population constantly increases with age.<sup>10</sup> In this context, our analysis of B19V IgG titers in plasma pools from recovered and source plasma that revealed a significant difference of mean B19V IgG titers may be caused by a difference in the age structure of plasma donors and blood donors. A B19V nucleic acid test (NAT) limit of less than 10<sup>5</sup> IU per mL B19V in plasma pools has been implemented by manufacturers of plasma derivatives as a voluntary standard some years ago.<sup>25</sup> Since the introduction of that test limit, no B19V transmission by plasma-derived products manufactured according to the standard has been observed, to our knowledge. The recent tendency toward tightening the B19V PCR test limit to less than 10<sup>4</sup> IU per mL, which would require interdicting donations that contain lower levels of B19V but may also contain B19V IgG, carries the possibility of also resulting in lower concentrations of B19V IgG in plasma for fractionation.<sup>6</sup> A direct comparison of plasma pools for which a B19V NAT test limit of 10<sup>4</sup> IU per mL has been implemented by regulatory requirement (anti-D plasma<sup>26</sup>) with plasma pools tested to comply with the Plasma Protein Therapeutics Association voluntary B19V PCR testing standard of less than 10<sup>5</sup> IU per mL (US source plasma)