Characterization of Markers of the Progression of Human Parvovirus B19 Infection in Virus DNA-Positive Plasma Samples

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Keywords

Antibodies · Blood safety · Donation deferral period · Immune response · Parvovirus B19 · Plasma donation · Transfusion-associated infections

Summary

Background: Accurate characterization of the infection stage in parvovirus B19(B19V)-positive plasma donations would help establish the donation deferral period to contribute to a safe fractionation pool of plasma. **Methods:** Viral DNA load of 74 B19V DNA-positive plasma samples from whole blood donations was determined by titration using nucleic acid testing. Markers of cellular (neopterin) and humoral (B19V-specific IgM and IgG) immune response were determined by ELISA in 32 B19V DNA-positive samples and in 13 B19V DNA-negative samples. The infection progression profile was estimated according to B19V DNA load and the presence of immune response markers. **Results:** B19V DNA load in the 74 samples was 10^6 – 10^{13} IU/ml. The distribution of 14 out of 32 selected B19V DNA-positive samples plus 2 B19V DNA-negative samples with no immune response marker followed along an upward curve according to B19V DNA load. After the peak, the distribution of 18 immune marker-positive samples followed along a downward curve according to their B19V DNA load and was grouped as follows: neopterin ($n = 4$), neopterin+ IgM ($n = 8$), neopterin + IgM + IgG ($n = 3$), IgM + IgG ($n = 2$), IgM ($n = 1$). There were 11 B19V DNA-negative IgG-positive samples. **Conclusion:** This study of B19V-DNA load and levels of neopterin, IgM, and IgG allows for reliable characterization and distribution into the different stages of B19V infection.

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Introduction

Parvovirus B19 (B19V) is an Erythrovirus (genus) of the Parvoviridae family. B19V has a tropism to the progenitor cells of erythrocytes and replicates in erythrocyte precursor cells in the bone marrow. Although virus replication is associated with a cytopathic effect [1], the majority of B19V infections take a clinical asymptomatic course; however, in some patient groups (e.g., pregnant women, patients with hemophilia, immunodeficient patients, and fetuses) B19V infection may take a more severe course [2, 3]. B19V is a prevalent worldwide infection common in humans. The incidence in young age groups (<15 years old) is approximately 50%, and in the elderly (>80 years old) the prevalence increases to 80% [4, 5].

B19V infection evolution is characterized by a 5-day phase with high viremia (titer about 10^{14} IU/ml B19V DNA) [6–8]. This viremia is neutralized with antibodies generated by the humoral immune system directed against two structural viral proteins VP1 and VP2 [9, 10]. The viremia decreases with the synthesis of immunoglobulin A (IgA) and immunoglobulin M (IgM), followed by the synthesis of immunoglobulin G (IgG) anti-B19V. IgA antibodies are detectable for a short period following the onset of clinical symptoms [11]. IgM antibodies are detected late in the viremic stage – about day 10–12 with a peak at day 15–22 – and can persist several weeks or months after the acute infection [12–15]. IgG antibodies replace IgM in the humoral immune response. They are detectable about 15 days post infection, with a peak at day 35–40 [16]. They remain high for several months and persist in the long term [12, 17].

The cell-mediated immune response occurs before the humoral immune response with the proliferation of specific CD4+ T cells against the VP1 and VP2 antigens. When the cellular immune response is activated, neopterin levels (6-D-erythro-trihydroxypro-

pilpiterin) increase also in B19V infection [18]. Neopterin is a direct marker for monocyte activity and an indirect marker for macrophage activity [19].

High titers of B19V in infected plasma or blood donors are considered a potential risk factor for B19V transmission via the blood or plasma product [20]. Aside from discarding donations with high-titer B19V, several safety measures have been developed to reduce the risk of viral transmission in plasma-derived products. Safety measures such as dry- or wet-heat treatment [21–24] and nanofiltration through small pore sizes (20 nm or lower) [25] are able to inactivate or remove B19V and other small non-enveloped viruses resistant to commonly used inactivation methods such as solvent/detergent (S/D). In addition, the European Pharmacopoeia in 2004 [26] recommended to blood product manufacturing industries that the viral load of B19V in manufacturing pools should not exceed 10^4 IU/ml for specific products [27]. In 2009, the US Food and Drug Administration (FDA) issued a guidance proposing the use of B19V nucleic acid technique (NAT) testing as an in-process test and the same viral load limit for manufacturing pools [28]. The objective of this requirement is to reduce the B19V load in manufacturing pools to levels that have been shown not to transmit infection in S/D-treated plasma.

The period between two donations by plasmapheresis donors can be very short. Since B19V-positive donors are usually allowed to give subsequent donations as long as they have the IgG but there is no active viremia, a reliable determination of the stage of the infection in a high-titer B19V donor would establish a donation deferral period while optimizing blood banking or plasma collection resources.

The objective of this pilot study was to characterize the B19V DNA-positive and -negative plasma samples according to their B19V DNA load and the presence of immune response markers to estimate a profile of the B19V infection progression.

Material and Methods

All plasma samples came from whole blood donations from routine analysis between 2006 and 2012. The B19V DNA-positive samples were discarded from fractionation, stored, and registered in the B19V Positives/Reactivities Library of Grifols. All studied B19V DNA-positive and -negative samples were negative for HAV RNA, HBV DNA, HCV RNA, and HIV-1 RNA, as well as for anti-HCV and anti-HIV1–2 antibodies and HBsAg antigen.

Study Design

The B19V DNA load of 74 known B19V DNA-positive samples was determined by titration, and samples were classified in categories of B19V DNA load. Of these, a smaller group of 32 samples was taken in proportion to each B19V DNA load category for cellular immune response determination (neopterin) and humoral immune response determination (IgM and IgG). Negative B19V DNA plasma samples were also tested ($n = 13$). Negative controls were obtained from a pool of plasmas.

Titration of B19V DNA in Plasma Samples

Direct detection by titration of B19V DNA (2 runs per sample) using NAT [29–33] was performed using the PROCLEIX® Parvo/HAV Assay from Logistic (Bedford, MA, USA) on the PROCLEIX® TIGRIS® System (Grifols Diagnostic, Barcelona, Spain), following the manufacturer instructions.

The PROCLEIX Parvo/HAV Assay has three main steps, which take place in a single tube on the PROCLEIX TIGRIS System: sample preparation, target amplification by transcription-mediated amplification (TMA), and detection of the amplification products (amplicons) by the hybridization protection assay (HPA). These assay steps are fully automated on the PROCLEIX TIGRIS System. An internal control (IC) RNA probe is added to each calibrator, test specimen, and control tube. The IC controls the specimen processing, amplification, and detection steps. Calibrator values for the PROCLEIX Parvo/HAV Assay are determined based on a comparison to the WHO International Standard for B19V DNA (Gen-Probe, Inc. PROCLEIX Parvo/HAV Assay 503805EN.RevA).

The IC and the HAV RNA probes have a rapid emission light (termed a 'flasher signal'), while the B19V DNA probe has a relatively slower kinetics of light emission (termed a 'glower signal'). Detection of the amplified products starts with the HPA, which is used to differentiate between hybridized and unhybridized probes by inactivating the label on unhybridized probes. Afterwards, the dual kinetics assay method is used to differentiate between the signals from flasher (IC and RNA-HAV) and glower labels (B19V DNA).

To assign a B19V DNA load quantitative value in international units (IU/ml), the glower signal corresponding to detection of B19V DNA is compared to a logistic dose-response calibration curve generated from the results of B19V DNA calibrator samples (with known concentration) included in each run. The PROCLEIX Parvo/HAV Assay determines quantitative results for B19V DNA load over a range from 5×10^2 – 10^5 IU/ml.

For obtaining a B19V DNA load within the dynamic range of the assay (5×10^2 – 10^5 IU/ml), experimental samples were diluted in negative plasma to prepare a dilution bank covering the possible range of B19V DNA load. From the serial dilutions, the $1:10^3$, $1:10^6$, and $1:10^9$ were analyzed for titer. Knowing the titer of those dilutions allowed experimental samples to be diluted to obtain 10^4 IU/ml and 10^3 IU/ml of B19V DNA load. Samples were then re-analyzed to confirm titer value.

Neopterin Levels

Neopterin levels were determined by ELISA using the IBL-International kit (IBL, International, Hamburg, Germany) following the manufacturer's instructions. This method is based on a competitive enzyme immunoassay in which an unknown amount of neopterin in the sample and a fixed amount of enzyme-labeled neopterin compete for the antibody binding sites [10, 16].

Neopterin analysis was initiated by adding 10 µl each of standard, control, and plasma sample into the respective wells of the microtiter plate. Afterwards, 100 µl of enzyme conjugate (neopterin conjugated to peroxidase) and 50 µl of neopterin antiserum (rabbit anti-human neopterin serum) were added to each well. Then, the microplate was incubated for 90 min at room temperature (18–25 °C). Following incubation, the microplate was washed 4 times with 300 µl of wash buffer, and 150 µl of substrate (tetramethylbenzidine (TMB)) was added to each well. After a second incubation of 10 min at room temperature, 150 µl of stop solution (sulfuric acid) was added to each well. The color intensity of each well after the substrate incubation is inversely proportional to the amount of antigen in the sample. Optical density was measured with a photometer at 450 nm.

Results were extrapolated by comparing the optical densities of the samples to a calibration curve obtained from the standards, which was previously adjusted with a four-parameter logistics test. Results were considered neopterin-positive if the neopterin level was ≥ 10 nmol/l and were considered neopterin-negative if the neopterin level was < 10 nmol/l.

IgG and IgM Antibody Levels

Assessment of B19V-specific IgG and IgM antibody levels was performed using two sandwich enzyme immunoassays (EIA) (Parvovirus B19 IgM and IgG Enzyme Immunoassay kits from Biotrin® (Dublin, Ireland)) following the manufacturer's instructions.

Determining the level of IgM started with the addition of 100 µl of the kit's positive control, external positive control, negative control, calibrator, and samples to their appropriate wells. Then, the microplate was incubated for 1 h at room temperature (18–25 °C) and then washed 4 times with 250 µl of wash solution. After the wash, 100 µl of biotin VP2 was added to each well; then the

Table 1. Number and percentage of the B19V DNA-positive quantified samples according to titer categories

Titer, IU/ml	Total samples, n (%)	Selected samples, n (%)
10 ⁶	25 (34)	7 (22)
10 ⁷	9 (12)	4 (13)
10 ⁸	8 (11)	4 (13)
10 ⁹	12 (16)	4 (13)
10 ¹⁰	7 (9)	4 (13)
10 ¹¹	7 (9)	4 (13)
10 ¹²	4 (5)	3 (9)
10 ¹³	2 (3)	2 (6)
Total	74 (100)	32 (100)

microplate was incubated for 30 min at room temperature and washed again 4 times. Conjugate (streptavidin-HRP) and the substrate (TMB) were then added. When the last incubation was complete, 100 µl of stop solution was added to each well. Optical density was measured with a photometer at 450 nm.

The protocol to assess IgG levels was identical to that of IgM with the exception of the biotin VP2 addition and the corresponding incubation and washing steps. The substrate and the stop solution were the same as the ones used for IgM. However, the conjugate used for IgG was a rabbit anti-human IgG HRP.

The results are expressed in a ratio of O.D. sample / O.D. cutoff. A ratio > 1.1 indicates the presence of IgM or IgG, a ratio < 0.9 indicates the absence of IgM or IgG, and a ratio between 0.9 and 1.1 is inconclusive.

Data Analysis

For quantitative variables, mean and range (minimum and maximum) were used. For qualitative variables, absolute and relative frequency in percentage is presented. Since this was a pilot study, sample size was not statistically pre-determined. To obtain a minimum of 2 samples in each B19V DNA load category, a size of 75 samples was estimated (final size, N = 74). To assess the immune response markers, a smaller group (40–50% of the 74 B19V DNA-positive samples) was taken (n = 32). These samples were randomly selected from each category of B19V DNA load of the whole initial set of 74 samples so that to parallel as close as possible the percentages obtained in each category, starting from a minimum of 2 samples. B19V DNA-negative samples of approximately 40–50% of this size were also assessed for the same markers (n = 13).

The 32 B19V DNA-positive and the 13 B19V DNA-negative samples were arranged along an estimated hypothetical curve according to the B19V DNA load (IU/ml in ordinate axis) and the type of marker(s) detected (time of antibody response in abscissa axis), resulting in a profile of the evolution of B19V infection [6, 8, 11, 12, 14, 16, 17].

Results

B19V DNA Load of Plasma Samples

All 74 B19V DNA-positive samples contained a B19V DNA load higher than 10⁶ IU/ml. A wide range of B19V DNA load categories was observed (10⁶–10¹³ IU/ml). Table 1 shows the number of samples classified according to its B19V DNA load in IU/ml (log10) and the number of the positive samples selected for each category. The values obtained in 11 out of the 13 B19V DNA-negative samples were below the lower limit of the method's dynamic range for negativity (500 IU/ml). The other 2 samples showed a B19V DNA load slightly above the limit (747 and 805 IU/ml). Since those values are within the method variability,

Fig. 1. Distribution of the 32 B19V DNA-positive and 13 B19V DNA-negative samples in titer categories, according to the percentage of samples neopterin (+) and (-).

these two samples were considered B19V DNA-negative, but identified as borderline.

The 32 B19V DNA-positive samples selected for further assessment of cellular and humoral immune response markers are shown in table 1.

Presence of Cellular Immune Response Marker (Neopterin)

15 of the 32 (47%) B19V DNA-positive samples analyzed were positive for neopterin (levels ≥ 10 nmol/l) with a mean value of 18.8 nmol/l (range 9.7–43.9 nmol/l) (fig. 1). B19V DNA load of these samples was between 10⁶ and 10¹² IU/ml (including one borderline sample with neopterin levels of 9.7 nmol/l). The other 17 (53%) B19V DNA-positive samples were negative for neopterin (levels < 10 nmol/l) with a mean value of 6.7 nmol/l (range 4.7–8.5 nmol/l); all of them had a B19V DNA load between 10⁶ and 10¹³ IU/ml.

With the exception of the 10¹³ IU/ml B19V DNA load category, positive neopterin levels were found in all other B19V DNA load categories, from 67% (2/3) in 10¹² IU/ml samples to 57% (4/7) in 10⁶ IU/ml samples.

All neopterin levels obtained in the 13 B19V DNA-negative samples were negative (levels < 10 nmol/l) with a mean value of 5.2 nmol/l (range 3.1–7.3 nmol/l).

Presence of Humoral Immune Response Markers (IgG and IgM)

IgM was present (ratio > 1.1) in 14 of the 32 (44%) B19V DNA-positive samples with an average ratio of 7.7 (range 1.5–21.3). IgM was not present (ratio < 0.9) in the other 18 samples (56%) with an average ratio of 0.5 (range 0.4–0.6). B19V DNA load of these samples ranged between 10⁶ and 10¹¹ IU/ml.

IgG was present in 5 (16%) of the 32 B19V DNA-positive samples (including one borderline sample with a ratio of 0.9), with an average ratio of 2.8 (range 0.9–6.3). All these samples had a B19V

Fig. 2. Distribution of the 32 B19V DNA-positive and 13 B19V DNA-negative samples titer categories, according to the percentage of samples IgM (+) / IgM (-) and/or IgG (+) / IgG (-).

Fig. 3. Classification of the 32 characterized samples according B19V DNA load (titer) and presence of neopterin, IgM, and IgG to represent the infection progression as plotted in the dotted line (gray square: outlier neopterin-positive sample with B19V DNA load below expected; gray triangle: IgM-positive sample with low neopterin levels; asterisk: samples with B19V DNA load slightly above the lower limit of the method's dynamic range for negativity but within the method variability limits).

DNA load of 10^6 IU/ml (with simultaneous presence of IgM). IgG was not present (ratio < 0.9) in the other 27 (84%) B19V DNA-positive samples with an average ratio of 0.10 (range 0.1–0.6).

IgM or IgG was not present (ratio < 0.9) in 18 B19V DNA-positive samples (56%) with an average ratio of 0.5 (range 0.4–0.6).

None of the 13 B19V DNA-negative samples was positive for IgM, whereas 11 (85%) were positive for IgG (including the 2 borderline negative samples), with an average ratio of 5.6 (range 1.7–8.1). The remaining 2 samples were negative for both immunoglobulins.

Figure 2 shows the distribution of the samples in the B19V DNA load categories according to the presence or absence of IgM and/or IgG. None of the 5 samples with the highest B19V DNA load (10^{12} and 10^{13} IU/ml) had IgM and/or IgG. IgM was detected in samples with a B19V DNA load ranging from 10^6 to 10^{11} IU/ml.

Infection Progression Profile

The 32 B19V DNA-positive samples and the 13 B19V DNA-negative samples were plotted following the estimated hypothetical curve of infection progression as shown in figure 3.

There were 14 DNA-positive samples with no immune response marker, which were arranged in ascending order on the left side of the curve, according to their B19V DNA load. The 2 B19V DNA-negative samples that were negative for immune response markers were plotted leftmost. 18 DNA-positive samples were positive for one or more immune response markers, which were arranged in descending order on the right side of the curve, according to their B19V DNA load and grouped according to immune response marker as follows: neopterin only (4 samples, one of them with B19V DNA load below what would be expected; shown as a gray square in fig. 3), neopterin + IgM (8 samples), neopterin + IgM + IgG (3 samples), and IgM + IgG (2 samples). One sample in the low-range B19V DNA load (5×10^7 IU/ml) was IgM-positive but did not show high neopterin levels (gray triangle in fig. 3). The 11 B19V DNA-negative samples positive for IgG were plotted rightmost.

Discussion

The inclusion of effective steps for the inactivation or removal of small non-enveloped viruses such as B19V in plasma-derived therapeutic concentrates have greatly improved the safety margin [34, 35]. Still, the plasma industry applies additional systematic screening of all plasma for fractionation to discard high virus titer donations. NAT determines the presence of viral DNA in plasma. In contrast, serological testing of blood donors relies on the presence of detectable virus-specific antibodies produced several weeks after the initial infection. Studies have shown that NAT significantly reduces the transmission risk of pathogens in blood donations made during the seroconversion period [36].

To ensure a safe plasma pool, high-titer B19V DNA donors must undergo a temporary donation deferral until active viremia is not present anymore in the donation despite the presence of IgG. Therefore, a better knowledge of the B19V infection profile of the plasma donor population would help to establish a donation deferral period and provide a medical benefit through notification of B19V-positive donors who would subsequently know their stage of infection [37, 38]. In this study, B19V DNA-positive plasma samples from discarded units derived from the routine screening of plasma for fractionation were characterized according to their B19V DNA load and markers of the immune response (neopterin, IgM, and IgG) to determine a possible profile of B19V infection progression. These four indicators combined have not been previously investigated.

The association of B19V infection and neopterin levels has not been thoroughly studied. Studies performed by Schennach et al. [18, 39] demonstrated that the cellular immune response, detected by neopterin levels, appears a few days after B19V infection and several days before the humoral immune response is activated resulting in B19V-specific antibodies [18, 39]. The cellular immune response decreases concomitantly with B19V load and the increase of specific antibodies. For that reason, the period of cellular and humoral immune response overlap is short.

The 14 B19V DNA-positive samples with no immune response markers likely correspond to blood donations in the initial phase of pre-seroconversion when B19V DNA load had not yet peaked [40]. The percentage of donations in the pre-seroconversion phase (44%) is in agreement with the 35% presented by Thomas et al. [41]. The samples with highest B19V DNA load (10^{12} – 10^{13} IU/ml) were included in these 14 pre-seroconversion samples, which suggest that after B19V infection the virus achieves high concentrations in a short period of time, leading to an immune response.

Of the 18 B19V DNA-positive samples positive for immune response markers, the 3 samples with the highest B19V DNA load (3×10^{12} , 2×10^{12} , and 3×10^{11} IU/ml) were neopterin-positive but IgM- and IgG-negative, which is in agreement with the earlier cellular immune response after B19V infection [18]. It is possible that some (approximately 30–50%) of the IgM/IgG-negative samples with neopterin positivity and high B19V DNA load could be false-negatives due to the formation of immunocomplexes with B19V particles [42]. However, this effect would have a minor impact on the estimated hypothetical curve of infection progression since, at most, only one of the two neopterin-positive / IgM-negative samples in the range of 10^{12} IU/ml titer would be affected.

As B19V DNA load decreased (10^6 – 10^{11} IU/ml), there were 7 neopterin-positive samples positive for IgM, which is evidence of the activation of the humoral immune response [10]. Decline of IgM was followed by the presence of IgG antibodies in the samples. Positive IgG samples appeared in the lowest B19V DNA load (10^6 IU/ml), among which 5 B19V DNA-positive samples were positive for all the immune response markers (neopterin, IgM, and IgG). Finally, detection of IgG antibodies and no detection of IgM antibodies in the absence of B19V DNA likely indicate a past B19V infection.

There were 2 low B19V DNA load samples with low neopterin levels that were positive for IgM and/or IgG, indicating that those samples were likely in an advanced phase of viral infection when the humoral immune response is the only active response.

In a similar study, Kooistra et al. [43] analyzed 67 B19V DNA-positive samples with a B19V DNA load between 10^6 and 10^{13} IU/ml. They found that 70% of samples were IgM- and IgG-negative, 24% were IgM-positive and IgG-negative, and 6% were IgM- and IgG-positive. Our results (56%, 28% and 16%, respectively) are similar to those data.

Interestingly, one sample with high neopterin levels (IgM- and IgG-negative) showed an outlier profile with respect to B19V DNA load (2×10^9 IU/ml). A possible explanation is that in that donor there was a delayed activation of the humoral immune response due to a longer cellular immune response. However, high neopterin levels are not specific for B19V; therefore, the possibility that the observed immune response could be caused by a non-B19V-related co-infection cannot be ruled out.

The 13 B19V DNA-negative samples (including the 2 negative borderline samples) showed normal (low) neopterin levels as expected, but the 11 (85%) IgG-positive samples correlate with the levels found in the general population, where approximately 80% of the adult population is IgG anti-B19V-positive [44]. IgG is present in the fractionation pools where it may be helpful in neutralizing the small B19V concentrations that could be present [5, 45]. B19V DNA at low, undetectable concentrations can persist in blood donors for months or years while anti-B19V IgG remains detectable [46–48]. It is possible that the 2 samples borderline negative for B19V DNA had very low B19V DNA levels as the result of a past infection [12–15]. The two B19VDNA-negative samples with no immune response markers may be indicative of persons susceptible of being infected with B19V in the future.

In summary, this study of combined B19V DNA infection indicators of B19V DNA load and neopterin, IgM, and IgG levels allows characterization and distribution of B19V DNA-positive samples from plasma donors into the different stages of the infection. Following the peak of B19V DNA load, the cellular immune response is the first to activate, followed by the humoral immune response in two steps, synthesis of IgM and later IgG. All four indicators studied appear to be valid for a profile study of the evolution of B19V infection, and therefore important to establish a donation deferral period in the collection of plasma from plasmapheresis donors who have been previously B19V DNA-positive. However, more comprehensive studies are needed to corroborate the feasibility of the findings on a larger scale. In any case, the existing screening program of all plasma for fractionation reduces the likelihood that no single high-titer B19V donation could enter the fractionation plasma pool.

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Disclosure Statement

The authors are employees of Biomat (Grifols).

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