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**研究報告の概要**

パルボウイルスB19は，ヒトに対してのみ病原性をもつパルボウイルスであるが，急性感染後は赤血球前駆細胞で高濃度まで複製する。その時点で多くの場合は無症候であるので，高度に汚染した血液であっても血液供給される。その上，非エンベロープであるパルボウイルスB19は現在の方法では容易に不活性化されない。この研究では様々な細胞株のB19感染に対する感受性を比較し，中和抗体と同様にウイルス感染を検出する様々な方法を評価した。
UT7/Epo-S1細胞株は，B19感染に最も高い感受性があることが判明した。そしてこの株では，間接免疫蛍光法により容易にB19カプシドタンパクが染色された。最も高感度の感染症分析はRNA転写を検出するRT-PCRあるいは定量的RT-PCR法であった。また，RNA転写を検出するハイスループット分析法が開発され，血漿プールに含まれた感染ウイルスを高力価で検出できた。さらに本分析法を確認する手段として血清で中和抗体を検出した。その結果，抗B19抗体を含む血清でプレインキュベートしたB19ウイルス感染UT7/Epo-S1細胞株では，RNA転写が顕著に減少していた。ここで紹介された分析法は，現存するものより多くの利点を示している。

| 報告企業の意見 | 今後の対応 |
|---|---|
| 弊社血漿分画製剤の製造工程における各段階におけるウイルス除去効果は，パルボウイルス B19 のモデルとしてブタパルボウイルスを用いて確認されている。ヒトトランスフェリンでは 5.9log 以上，ヒトアルブミンでは 6.8log 以上，またヒト免疫グロブリンでは 9.3log までウイルス除去が可能である。したがって，弊社の血漿分画製剤では，ウイルス伝播のリスクは極めて低い。 | 現時点で新たな安全対策上の措置を講じる必要は無いと考える。引き続き関連情報の収集に努める。 |

**使用上の注意記載状況・その他参考事項等**

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# Development of an improved method of detection of infectious parvovirus B19

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**Abstract**

*Background:* Parvovirus B19, the only known pathogenic human parvovirus is the aetiologic agent of erythema infectiosum, transient aplastic crisis, pure red cell aplasia, and hydrops fetalis. Transmission is either by respiratory secretions or, as it can be present at high titre in plasma, by blood and blood products. B19 is only cultured with difficulty in vitro, and there is no readily available assay for detecting B19 infectivity or neutralizing antibodies.

*Objectives:* In this study, we evaluated different methods to detect viral infection for the purpose of developing automated methods for large-scale testing of viral infectivity, development of neutralizing antibody and viral inactivation assays.

*Study design:* Different cell lines were evaluated for their ability to support B19 infection and assays tested for sensitivity and ease of performing. A high-throughput assay was validated by determining infectious virus in blood pools and for determining neutralizing antibody in sera.

*Results:* B19 protein production was detected by immunofluorescence (IF) staining and increased viral DNA production by dot blot hybridization and quantitative PCR. The detection of RNA transcripts by RT-PCR assay and quantitative RT-PCR (qRT-PCR) was used as an indirect marker for infection. Of the cell lines tested, the subclone UT7/Epo-S1 showed the greatest sensitivity to B19 infection, with detection of viral transcripts by qRT-PCR the preferred assay. The assays were validated by experiments to determine the infectious titre of sera from acutely infected humans, to evaluate the presence of infectious virus in human donor plasma pools and to measure neutralizing antibodies.



*Keywords:* Parvovirus B19; Erythrovirus infections; Neutralizing antibody

## 1. Introduction

Parvovirus B19 is the only known parvovirus pathogenic to humans. It is associated with a number of diseases including erythema infectiosum ("fifth disease") in children, arthropathy commonly seen in women, transient aplastic crisis in individuals with high red cell turnover, pure red cell aplasia in immunocompromised patients, and hydrops fetalis following infection during pregnancy (Young and Brown, 2004).

B19 is highly erythrotropic and replicates to high titre in erythroid progenitor cells. In healthy individuals, at the height of the transient viraemia, viral titres as high as $10^{13}$ genome equivalents (ge)/mL are detectable. Individuals are often asymptomatic at this time, and highly viraemic blood donations do enter the blood supply. In addition, as the virus has only a small (5600 nucleotide) DNA genome, and is non-enveloped, the virus is relatively heat resistant (Schwarz et al., 1992) and not removed by solvent/detergent methods normally used to inactivate virus (Mortimer et al., 1983b; Sayers, 1994). Depending on the sensitivity of the assay, B19 DNA can be detected in between 0.11% and 0.003% of blood donor samples (Jordan et al., 1998; McOmish et al., 1993; Mortimer et al., 1983b; Tsujimura et al., 1995; Wakamatsu et al., 1999), and transmission of B19 infection to recipients of both blood

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and blood products has been frequently documented (Azzi et al., 1999). Attempting to reduce the risk of B19 infection has become a major concern for blood and blood product suppliers in Europe and America (Brown et al., 2001), with many countries now advocating screening of plasma pools to remove samples containing high B19 viral titres.

However, B19 DNA appears to be very stable and low levels of B19 DNA can persist in serum and a range of tissues for months following acute infection (Soderlund-Venermo et al., 2002), and detection of viral DNA does not equate necessarily with active viral infection. In addition, assays for detecting infectious virus are extremely limited. Although B19 has been shown to replicate in vitro in primary erythroid progenitor cells from bone marrow (Mortimer et al., 1983a), peripheral blood (Ozawa et al., 1986) and fetal liver (Brown et al., 1991; Yaegashi et al., 1989), very few cell lines have been found to be permissive for B19 infection, and even in these, viral replication is inefficient. The first cell line described to be permissive for B19 infection was an erythropoietin (Epo)-dependent subclone of UT7, a megakaryoblastoid cell line (Shimomura et al., 1992). Since then, a limited number of additional cell lines have been described, including KU812Ep6, an erythroleukaemic cell line (Miyagawa et al., 1999), and JK-1 cells (Takahashi et al., 1993). To date no comparative studies of the differences in susceptibility/sensitivity and permissivity amongst these cell lines have been published.

A number of different methods have been suggested for detecting parvovirus B19. Currently, methods for identifying active clinical B19 infections include detection of B19 nucleic acid testing by direct dot blot hybridization (Anderson et al., 1985) or PCR (Cassinotti et al., 1993; Clewley, 1992), and more recently RT-PCR for RNA transcripts (Wong et al., 2003). Similar methods have been used for detecting infection in cells or cell lines, but little has been published on the relative sensitivity of the different methods.

In this study we compared the susceptibility/sensitivity of various cell lines to B19 infection and evaluated different methods to detect viral infection. In addition, we established a high-throughput method for detection of B19 infection and validated the assay by using it to detect infectious virus in plasma pools and neutralizing antibodies in serum samples.

## 2. Materials and methods

### 2.1. Cell lines

Cell lines were obtained from American Tissue Type Collection (ATCC, Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum (FCS), penicillin, streptomycin and glutamine (P/S/G; Gibco/Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% $CO_2$ unless otherwise stated. UT7/Epo (Shimomura et al., 1992) were maintained in RPMI 1640 with 10% FCS and 5 U/mL of Epo (Amgen, Thousand Oaks, CA, USA). UT7/Epo-S1, a subclone of UT7/Epo and a gift from Dr Sagamura (Morita et al., 2001) were maintained in Iscove's modified Dulbecco's media (IMDM) plus 10% FCS, and 2 U Epo/mL. KU812Ep6, a gift from Dr Miyagawa (Miyagawa et al., 1999) were maintained in RPMI 1640, 10% FCS and 6 U Epo/mL K-562 and JK-1 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany) were maintained in RPMI 1640, with 10% FCS.

### 2.2. B19, serum and plasma sources

Plasma and serum samples containing high-titre infectious B19 was obtained from several sources. J35 was obtained from a sickle-cell patient in aplastic crisis, and previously determined to be negative for both B19 IgM and IgG (data not shown). Additional plasma samples were obtained from normal donors at the time of blood donation, and provided by Mei-Ying Yu at CBER, FDA and Aris Lazo at V.I. Technologies, Inc. The WHO standard sample (Saldanha et al., 2002) was obtained from National Institutes of Biological Standards and Control (NIBSC), South Mimms, UK.

Plasma pools containing high-titre B19 were a kind gift of Matthias Gessner of Baxter. Serum samples from laboratory donors and healthy blood donors obtained as part of the National Heart, Lung, and Blood Institute (NHLBI) Retrovirus Epidemiology Blood Donor Study, NHLBI repository, and previously tested for antibody to parvovirus B19 (Brown et al., 2004) were used in the neutralization assay.

### 2.3. Comparison of infectivity of different cell types

The infection assay was as previously described (Nguyen et al., 2002). Briefly, on the day before infection, cells were split 1:2 with appropriate media to induce cell division. On the day of infection, cells were seeded at $2 \times 10^5$ cells/100 μL, an equal volume of virus dilution was added and the infection was allowed to incubate at 4 °C for 2 h. After the incubation, 800 μL of appropriate media was added to the infection bringing the volume to 1 mL. The cells were transferred to a 24-well plate and incubated at 37 °C and harvested for analysis on Days 1, 2, 3, or 6.

### 2.4. Immunofluorescence (IF) assay for B19 capsid proteins

Cells were evaluated for B19 protein production by IF staining. Approximately $5 \times 10^4$ cells were collected onto glass slides by cytocentrifugation at 1500 rpm for 8 min and fixed in methanol–acetone (1:1) at −20 °C. Viral capsid proteins were detected by mouse anti-B19 monoclonal antibody, 521-5D (a gift from Larry Anderson, CDC, Atlanta, GA, USA) followed by goat anti-mouse IgG FITC (Zhi et al., 2004).

### 2.5. B19 DNA

DNA direct hybridization was used to quantitate the B19 copy number as previously described (Brown et al., 1994). Briefly, plasmid pYT103 was diluted to give a range of DNA concentrations (0, 0.1, 1, 10, and 100 pg/μl). Plasmid dilutions, serum samples or extracted DNA (10 μl) were added to 200 μl 0.333 M NaOH and incubated at room temperature to denature the DNA. The samples were then applied to a nylon membrane (0.45 μm pore, Nytran Plus, Schleicher and Schuell, Keene, NH, USA) using a dot blot manifold apparatus (96-well, Schleicher and Schuell), the membrane washed in 6× SSC, and the membrane baked at 80 °C for an hour in a vacuum oven. The membranes were hybridized with a $^{32}P$ random-primed probe of the complete B19 coding region (*Eco*RI digest of pYT103) in Hybrisol (Serologicals Corporation; 42 °C overnight). The membranes were washed with 2× SSC; 0.1% SDS at room temperature and 0.1× SSC; 0.1% SDS for 20 min at 55 °C, and then exposed to either a "phosphor" screen (Molecular Dynamics, GE Healthcare) or X-ray film.

### 2.6. Quantitative PCR

DNA was extracted from cells and supernatant using the QIAmp DNA mini Kit (Qiagen, Valencia, CA, USA), and quantitated by qPCR using the QuantiTect Probe PCR kit (Qiagen), using primers in the capsid region of the virus. Specifically the primers and probe used were B19-Cap-F (5′-TACCTGTCTGGATTGCAAAGC-3′; 0.4 μM) and B19-Cap-R (5′-GATGGGTTTTCTAGGGGATTATC-3′; 0.4 μM) and 0.2:M B19-Cap-Probe probe (6-FAM-ATG GTG GGA AAG TGA TGA TGA ATT TGC TA-Black Hole Quencher). Quantitation of the number of genome copies was estimated by comparison to a standard curve obtained from serial dilutions of the pYT103, and confirmed by testing the NIBSC standard (Saldanha et al., 2002).

### 2.7. RT-PCR

As previously described, an RT-PCR assay was used to look for spliced viral transcripts as a marker for infection (Nguyen et al., 2002). Briefly, cells were harvested and total RNA extracted using 200 μL RNA STAT-60. Contaminating DNA was removed using RQ1 DNAse (Promega) incubation for 15 min at room temperature and RNA was reverse transcribed by initially incubating RNA with random primers and reverse transcriptase, Superscript II (Invitrogen), prior to PCR amplication with primers B19-9 and B19-1. To increase detection of spliced products from the RT-PCR reactions, products were resolved on a 2.5% NuSieve agarose gel and southern hybridization was performed using probe labeled with alkaline phosphatase (CDP-Star AlkPhos labeling kit, Amersham).

### 2.8. Quantitative RT-PCR

RNA transcripts were quantitated by real-time RT-PCR designed to amplify products in the capsid and NS regions using the QuantiTect Probe RT-PCR kit (Qiagen). The QuantiTect Probe RT-PCR master mix and QuantiTect RT mix was combined with 0.4 μM of the amplification primers (NS primers 5′-GTTTTATGGGCCGCCAAGTA-3′ and 5′-ATCCCAGACCACCAAGCTTT-3′; capsid primers 5′-CCTGGGCAAGTTAGCGTAC-3′ and 5′-ATGAATCCTTGCAGCACTGTCA-3′), and 0.2 μM probe (NS probe FAM 6′-CCATTGCTAAAAGTGTTCCA-BHQ1; capsid probe FAM-TATGTTGGGCCTGGCAA-TAMRA). After an initial activation step of 15 min at 95 °C, 45 cycles of 15 s at 94 °C and 60 s at 60 °C were performed. Quantitation of the number of transcripts was by estimating the cDNA copy number by comparison of a standard curve of serial dilutions of pYT103 as described for the quantitative PCR.

To confirm extraction of RNA, and to normalize the number of transcripts per cell, quantitative RT-PCR (qRT-PCR) was performed using the same amplification conditions, but with primers β-actin F (5′-GGCACCC-AGCACAATGAAG-3′), β-actin R (5′-GCCGATCCACA-CGGAGTACT-3′) and actin probe (5′JOE-TCAAGA-TCATTGCTCCTCCTGAGCGC-3′BHQ). An actin standard curve was obtained from serial dilutions of a plasmid containing an extended region of the actin coding sequence.

### 2.9. High-throughput qRT-PCR

To develop a high-throughput method for detecting RNA transcripts, RNA was extracted from cells using GeneStrips™ (RNAture, Irvine, CA, USA) according to the manufacturer's protocols. mRNA extracted using this method was converted to cDNA using MMLV-RT (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocol scaled up to a 50 μL reaction volume. B19 RNA transcript production was determined by qRT-PCR as described above.

In some experiments the infection volume was scaled down to 100 μL and incubated in 96-well plates.

### 2.10. Detection of infectious virus in plasma pools

Plasma pools of 2000 donors that contained B19 DNA by PCR B19 were tested for their ability to infect UT7/Epo-S1 cells. Infection was as described (100 μL infection volume) and RNA was extracted with RNAture Genestrips and analyzed by qRT-PCR. The number of B19 DNA copies in the original plasma pools was determined by quantitative PCR.

### 2.11. Neutralizing antibody detection

Detection of neutralizing antibodies was assayed by qRT-PCR. Serum or plasma from donors was incubated with serial dilutions of high-titre B19 containing serum for 1 h prior to

infection with UT7/Epo-S1 cells as described for infections (100 μL in a 96-well plate). RNA was extracted from cells using the RNAture Genestrips and analyzed by qRT-PCR.

## 3. Results

### 3.1. Comparison of sensitivity and permissiveness in haematopoietic cell lines

The majority of the haematopoietic lines tested (HL-60, HEL, KG-1, KG-1a, K-562, U-937) were negative by both IF for capsid protein and detection of spliced transcripts indicating that the cells were non-permissive. Only UT7/Epo cells, KU812Ep6, the UT7/Epo-S1 subclone and JK-1 cells showed evidence of B19 infection with the UT7/Epo-S1 cells having the greatest sensitivity. However, by IF, the number of positive cells was always low, with <1% positive staining for KU812Ep6, UT7/Epo and JK-1 cells, but approximately 15% positive staining for UT7/Epo-S1 cells. This greater sensitivity was confirmed by detection of transcripts in UT7/Epo-S1 cells at $10^4$ ge/infection of the high-titre serum, 3 logs lower than that detected in the other cell lines. Subsequent studies were all done with the UT7-Epo-S1 cells.

### 3.2. Comparison of sensitivity of different methods to detect infectious B19

IF staining for B19 capsid protein production detected infected cells consistently at $10^{-2}$ to $10^{-3}$ dilutions of high-titre serum (>$10^{12}$ ge/mL), but the number of positive cells was low (15–1%). Determining viral DNA production by direct hybridization of viral DNA by dot blot was limited to detection above $10^9$ ge/infection, in part because only 10 μL of sample was analyzed from a 1 mL infection. In time course experiments, when infecting with high concentrations of virus ($10^9$ ge/mL) spliced transcripts could be detected on the first day, rising on the third day. When samples were tested on Day 3, the most sensitive methods were those detecting RNA transcripts, either by conventional or qRT-PCR assay or detecting the increase in viral DNA production by qPCR (Table 1).

Table 1
Comparison of the different sensitivities of assays used to determine B19 infectivity

| Detection method | Sensitivity, genome equivalents (ge) |
|---|---|
| Protein: IF | ~$10^8$ |
| DNA: dot blot hybridization | $10^9$ |
| DNA: quantitative PCR | $10^4$ |
| RNA: RT-PCR | $10^4$ |
| RNA: quantitative RT-PCR | $10^4$ |

UT7/Epo-S1 cells were infected with dilutions of B19, and cells assayed on Day 3. Results are the minimum amount of virus added to a 1 mL cell culture to detect infectivity.

Table 2
Comparison of the number of infectious units of B19 to the viral DNA in different serum or plasma samples

| Viral stock | Genome equivalents ((ge)/mL × $10^{12}$) | Infectious (units/mL) | ge/infectious units |
|---|---|---|---|
| J35 | 33.2 ± 2.0 | $10^8$ | $3.3 \times 10^5$ |
| VS2 | 10.2 ± 2.9 | $10^9$ | $1.0 \times 10^4$ |
| V1 | 2.0 ± 0.3 | $10^8$ | $2.0 \times 10^4$ |
| V2 | 2.9 ± 0.2 | $10^8$ | $2.9 \times 10^4$ |
| V3 | 0.1 ± 0.1 | $2 \times 10^7$ | $5 \times 10^3$ |
| CBER STD | 1.0 | $10^8$ | $1.0 \times 10^4$ |

Viral DNA measured by qPCR. UT7/Epo-S1 cells were infected with serial dilutions of virus, on Day 3 RNA was extracted with RNA STAT60 and B19 transcripts detected by RT-PCR.

### 3.3. Comparison of the infectivity of different serum samples with viral copy number

Serial dilutions of different viral sera stock were used to infect UT7/Epo-S1 cells, and the infectious titre determined by the endpoint of detection using RT-PCR (Table 2). The viral genome equivalents in each sample was determined for each sample by qPCR and confirmed by dot blot analysis and the ratio of viral DNA (ge) were compared to infectious units. The ratio of genome equivalents were relatively high compared to infectious units, with ratios ranging from $2 \times 10^5$ ge in the J35 stock to $5 \times 10^3$ ge per infectious unit in the CBER standard.

### 3.4. Comparison of RNA extraction methods

Cell lysates were directly incubated in RNAture "Genestrips", washed off, and cDNA synthesized in situ, prior to qPCR. In direct comparion tests RNA extracted from scaled down 0.1 mL cultures with RNAture Genestrips showed comparable sensitivity to 1 mL cultures extracted with RNA STAT-60 (Table 3).

Table 3
Equivalent sensitivity of detection of infectious virus using two different methods of RNA extraction and B19 transcript detection

| Virus dilution | Standard method | | High-throughput method | |
|---|---|---|---|---|
| | Day 0 | Day 3 | Day 0 | Day 3 |
| $10^{-5}$ | – | +++ | 0 | 27,345 |
| $10^{-6}$ | – | +++ | 0 | 1328 |
| $10^{-7}$ | – | +++ | 0 | 231 |
| $10^{-8}$ | – | – | 0 | 1 |
| $10^{-9}$ | – | – | 0 | 0 |

A plasma stock (V1) was serially diluted in culture medium and used to infect UT7/Epo-S1 cells. Standard method, RNA was extracted from a 1 mL culture using RNA STAT-60 and transcripts detected by RT-PCR. +++, bands easily detected by ethidium bromide staining and with an alkaline phosphatase-labeled specifc probe; –, bands not detected; and high-throughput method, RNA was extracted from a 0.1 mL culture using RNAtureGenestrips and transcripts detected by NS qRT-PCR. Quantitations are given as ge/μL of RT reaction volume.

Table 4
Detection of infectious B19 in plasma pools

| Sample # | Stock (ge)/mL × $10^{12}$ | RT-PCR result | VP qRT-PCR (ge/infection) × $10^8$ | NS qRT-PCR (ge/infection) × $10^8$ |
|---|---|---|---|---|
| Pool #1 | 1.8 ± 0.4 | +++ | 2.1 ± 0.9 | 2.2 ± 1.4 |
| Pool #2 | 3.2 ± 0.1 | +++ | 4.6 ± 0.8 | 3.7 ± 0.9 |
| Pool #3 | 0.5 ± 0.1 | +++ | 2.8 ± 0.3 | 2.1 ± 0.4 |
| Pool #4 | 0.5 ± 0.04 | +++ | 2.6 ± 0.9 | 2.4 ± 0.5 |
| Pool #5 | 0.005 ± 0.0005 | + | 0.0023 ± 0.001 | 0.0036 ± 0.002 |
| Pool #6 | 1.1 ± 0.3 | +++ | 3.0 ± 0.2 | 1.4 ± 0.05 |
| No virus | 0 | – | 0 | 0 |

The amount of B19 in six plasma pools (previously known to contain B19) was determined by qPCR and the presence of infectious virus was determined by infection of UT7/Epo-S1 cells and detection of RNA transcripts after by either RT-PCR or using RNAture Genestrips and qRT-PCR amplifying the capsid (VP) and nonstructural (NS) regions.

### 3.5. Detection of infectious virus in pooled blood products

To test the sensitivity of our high-throughput assay and to test its potential application for screening plasma pools for infectious virus we examined six plasma pools previously identified as containing high-titre B19 DNA. qPCR analysis confirmed that all samples contained B19 DNA, with five of the six samples containing high B19 titres with >$10^{11}$ ge/mL. The sixth sample had ~5 × $10^9$ ge/mL. However, after infection of UT7/Epo-S1 cells, RNA transcripts could be detected by both RT-PCR and qRT-PCR, indicating that all the pools contained infectious B19 (Table 4).

### 3.6. Neutralization

As further validation of the high-throughput assay the method was used to detect B19 neutralizing antibodies in six sera of known B19 antibody status. After incubation with serial dilutions of each serum with virus for 2 h, the antibody/virus complex was allowed to infect UT7/Epo-S1 cells in a microtitre plate, RNA extracted at Day 3 RNAture Genestrips, and qRT-PCR performed. No block of infection was detected in the two sera that were B19 IgG negative (Table 5). In contrast, at the highest concentration of virus, there was a marked reduction in the number of viral transcripts with all four sera. If a reduction of viral transcripts by >90% is considered the endpoint, then in two sera the neutralizing titre was >$10^5$, and in the other $10^3$ and $10^4$, respectively.

## 4. Discussion

Although the erythroid tropism and inhibition of erythroid colony formation was demonstrated in 1983 (Mortimer et al., 1983a) and replication in vitro infection of bone marrow was demonstrated in 1986 (Ozawa et al., 1986), there is still no readily available method for culturing parvovirus B19 in the laboratory, limiting both the virus availability and the ability to develop assays to determine B19 infectivity. Similarly there are no readily available methods for detecting neutralizing antibodies in patient samples, or for testing viral inactivation procedures for blood and blood products.

A number of cell lines have been described that support B19 infection, and a number of infectivity assays have been described, based on these cell lines. Specifically, infection and neutralization assays based on UT7/Epo cells (Bostic et al., 1999), KU812Ep6 (Blumel et al., 2002; Bonvicini et al., 2004; Miyagawa et al., 1999; Saito et al., 2003) and UT7/Epo-S1 (Prikhod'ko et al., 2005) cells have all been described. More recently, cells that are not fully permissive for B19 infection have also been evaluated (Caillet-Fauquet et al., 2004) However, there have been no attempts to compare the different cell types or sensitivity of the different methods. In addition, many of the methodologies are very labour intensive, and/or require reading of IF, and are therefore not readily automated or applicable to testing large numbers of samples.

In our study, IF was the least sensitive of the methods, with apart from UT7/Epo-S1 cells, generally less than 1% of cells being positive even after inoculation with high-titre virus.

Table 5
Neutralizing antibody assay using quantitative RT-PCR in the B19 NS region

| Serum sample | 4 | 5 | 44 | 45 | A | B |
|---|---|---|---|---|---|---|
| IgG | + | + | – | + | + | – |
| Serum alone | 0 | 0 | 9 | 0 | 0 | 8 |
| B19 + $10^{-3}$ serum | 189 | 0 | 5244 | 0 | 0 | 7393 |
| B19 + $10^{-4}$ serum | 30,215 | 3 | 14,423 | 50 | 34 | 5801 |
| B19 + $10^{-5}$ serum | 40,657 | 64 | 68,165 | 423 | 12,674 | 79,557 |
| B19 alone | 30,027 | 20,709 | 24,234 | 19,666 | 11,448 | 20,676 |
| Cells only | 0 | 0 | 0 | 0 | 1 | 2 |

Normal donor serum was preincubated with dilutions of high-titre B19 plasma (>$10^{12}$ ge/mL) and used to infect UT7/Epo-S1 cells. A representation of quantitative data obtained is shown and given in ge/μL of RT reaction volume and normalized against the qPCR obtained for β-actin.

This percentage of positive cells was lower than published in the literature: Miyagawa reported KU812Ep6 cells as having about 30% of the cells positive for B19 infection (Miyagawa et al., 1999), and Morita reported that 40% of the UT7/Epo-S1 stained positive for B19 infection (Morita et al., 2001). Some of these discrepancies may be due to the amount of virus used for the infection (at higher titre we can observe that 30% of cells are positive), differences in culture techniques, and the specificity of the antibody used (Mab 521-5d used in these studies is specific for capsid conformational epitopes). However, as reading IF slides is not readily automated we did not spend more time optimizing the method.

Assays based on RT-PCR were the most sensitive assays, and in contrast to DNA-based assays were not confounded by input viral genomes. As with the IF assay, they also confirmed that the UT7/Epo-S1 cells were the most sensitive cell line, and using these cells, we could detect infectious virus with inoculums of $\sim 10^4$ genome copies. This is also in keeping with other published results that suggest the ratio of infectious virus:genome copies is 1:10,000 (Bonvicini et al., 2004; Miyagawa et al., 1999), not dissimilar to that of other *Parvoviridae* (Tattersall and Cotmore, 1988).

Due to the concerns of B19 contamination in blood and blood product, there is currently great interest in not only developing methods to detect infectious virus, but also to evaluate methods of viral inactivation/removal. Due to the difficulty in working with B19, many inactivation studies have been undertaken using a surrogate parvovirus, normally porcine parvovirus. However, when comparisons have been undertaken, porcine parvovirus and B19 have different properties as far as heat inactivation (Blumel, 2004) and pH stability (Boschetti et al., 2004), suggesting where possible the model virus should be studied. Although we, with colleagues, have previously described infections assays based on UT7/Epo cells by detection of spliced transcripts (Bostic et al., 1999; Lazo et al., 2002) or quantitative PCR (Prikhod'ko et al., 2005), we believe that the combination or RNA extraction process with quantitative RT-PCR is the easiest method for detecting B19 infection under a wide range of different clinical and experimental conditions.

Finally, the detection of neutralizing antibodies for parvovirus B19 continues to be challenging. Although methylcellulose-based assays were originally described, they are insensitive, and require large amounts of infectious virus, and are quite labour-intensive both to set up and to read. In contrast, the assay described here, requires small amount of B19 virus, and can be readily set up in a microtitre plate format. In some patients, especially immunocompromised patients, who have low levels of DNA in serum or tissues in the presence of B19 IgG, the decision as to whether treatment with IVIG would be beneficial can be difficult. Measurement of neutralizing antibodies in these circumstances would be helpful. In addition, such assays will be critical in determining the response to B19 immunization when the B19 vaccine becomes available.

## 5. Conclusions

This assay can be used to determine the infectious titre of parvovirus B19 in a number of different settings. In addition, the ability to automate many of the steps in the assay may allow this assay to be used more widely than is currently available.

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別紙様式第2-1

No. 8

# 医薬品 研究報告 調査報告書

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**研究報告の概要**

○パルボウイルスB19暴露に対する抗体陰性および陽性の人における免疫反応
ウイルスが混入した血液製剤を投与された後のパルボウイルスB19に対する免疫反応に関する情報はほとんど得られていない。最近の研究で、B19DNAを含む(1.6x10$^8$IU/mL)プール血漿の輸血後、B19抗体陽性の患者のB19IgG抗体のレベルが19-39 IU/mLから50-100 IU/mLまで上昇して再感染を防いだことを発見した。B19抗体陰性の患者における1.6-2.2 x 10$^8$IU/mLのB19DNAの存在は、プール血漿のIgGレベル59.5 IU/mLではB19の伝播とそれに続くセロコンバージョンを防ぐのは不十分であることがわかった。これらのデータは血液製剤の安全性評価の進歩につながるだろう。

| 報告企業の意見 | 今後の対応 |
|---|---|
| B19DNAを含む(1.6x10$^8$IU/mL)プール血漿の輸血後、B19抗体陽性の患者では抗体価が上昇して再感染を防いだが、陰性の患者における1.6-2.2 x 10$^8$IU/mLのB19DNAの存在は、プール血漿のIgGレベル59.5IU/mLではB19の伝播とセロコンバージョンを防ぐのは不十分であることがわかったとの報告である。 | 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。今後は検査方法の改善によりさらなる感度向上を目指すこととしている。 |

**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



13

MedDRA/J Ver.9.0J

BRIEF REPORT

# The Immune Response to Parvovirus B19 Exposure in Previously Seronegative and Seropositive Individuals

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**Little information is available on the immune response to parvovirus B19 after the administration of contaminated blood products. In the present study, we found that levels of B19 IgG in B19-seropositive recipients protect against reinfection and, after transfusion with pooled plasma containing B19 DNA ($1.6 \times 10^8$ IU/mL), increase from 19–39 IU/mL to 50–100 IU/mL. We found that, in the presence of $1.6–2.2 \times 10^8$ IU of B19 DNA/mL in B19-seronegative recipients, a pooled-plasma B19 IgG level of 59.5 IU/mL is insufficient to prevent B19 transmission and subsequent seroconversion. These data should lead to improvements in the assessment of blood-product safety.**

Parvovirus B19 can cause severe disease in immunocompromised individuals, and B19 infection during pregnancy can lead to fetal mortality. B19 infection is transmitted either via respiratory secretions or via administration of contaminated blood or blood products. The latter mode of transmission is especially problematic because of the high resilience of B19 to many of the treatments used in plasma processing, such as solvent-detergent treatment, lyophilization, and high temperatures [1], and also because of the extremely high levels of viremia in acutely infected, and often asymptomatic, individuals (>$10^{12}$ B19 DNA genome equivalents [GE]/mL or IU/mL) [2].

Significant efforts to minimize the B19 viral load in blood products commenced in the late 1990s because of the advent of robust DNA extraction and B19 polymerase chain reaction methodologies in addition to cases of B19 seroconversion in healthy volunteers who received contaminated plasma as part of a postmarketing surveillance study [3]. Most manufacturers now undertake minipool B19 nucleic acid testing to reduce plasma-pool levels of B19 DNA to <$10^4$ IU/mL, to conform with US Food and Drug Administration (FDA) proposals (available at: http://www.fda.gov/). Standardization of B19 DNA and IgG quantitation, as well as the establishment of validated serological assay systems, has also contributed to improvements in blood-product screening paradigms. The regulatory requirement that levels of B19 DNA in anti-D antibody preparations be <$10^4$ IU/mL [4] further illustrates the actions taken by regulatory agencies to effectively improve blood-product safety.

In the future, because of enhanced screening protocols, B19 transmission after the administration of blood products should become a less frequent event. However, heightened awareness of B19 has resulted in the emergence of relevant information regarding the infectious dose of B19 and the role played by B19 IgG in attenuating transmission. Koenigbauer et al. [5] reported a case of B19 infection in a 36-year-old woman that resulted from administration of a solvent/detergent-treated pooled plasma that was subsequently recalled by the American Red Cross after high levels ($10^7–10^8$ GE/mL B19 DNA) of B19 DNA were detected by the manufacturer. Blumel et al. [6] detailed 2 cases of B19 infection resulting from the administration of B19 $IgG^-$ plasma protein–complex concentrates: 1 individual received 180 mL of heat-treated concentrate containing $8.6 \times 10^6$ GE of B19 DNA/mL ($1.5 \times 10^9$ GE total), and the other received 996 mL of material containing $4 \times 10^3$ GE of B19 DNA/mL ($3.9 \times 10^6$ GE total). The transmission of B19 by a factor VIII concentrate (free of B19 IgG) has been documented in a case in which seroconversion occurred as a result of infusion of $2 \times 10^4$ IU of B19 DNA ($1.3 \times 10^3$ IU/mL) [7]. Solvent/detergent-treated plasma (Plas+SD) has also been identified, subsequent to a postmarketing surveillance study of this product, as the source of B19 infection that occurred in 18 individuals [3, 8]. It was concluded that B19 IgG in pooled plasma (64.7 ± 17.5 IU of B19 IgG/mL; [9]) was not protective in the presence of high B19 viral titers ($10^7–10^8$ GE/mL) and that plasma lots containing low viral titers ($10^{0.5}–10^{3.5}$ GE/mL) did not cause B19 infection in plasma recipients. However, detailed serological analysis of this event has not been forthcoming, and the significance that the data have for wider issues of

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

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

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

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

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

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

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

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

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

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

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

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

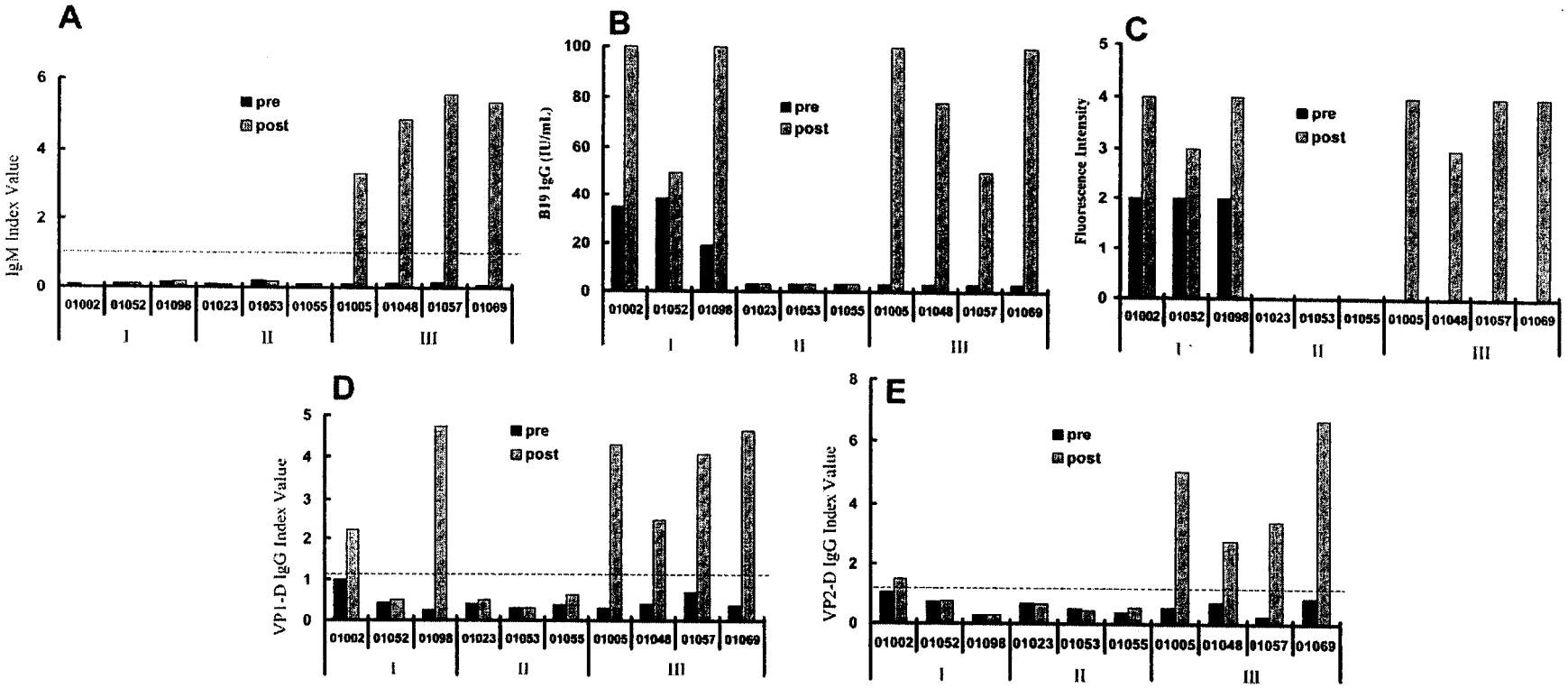


**Figure 1.** *A,* B19 IgM reactivity against conformational VP2, in pooled-plasma recipients (before and after transfusion). Reactivity is measured as IgM index value (reactivity >1.1 is reactive *[dashed line]*). *B,* B19 IgG reactivity against capsid (conformational) VP2 (VP2-N), as determined by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

epitopes on the unique region of VP1 increase dramatically. Moreover, it also proposes that the levels of B19 IgG in pooled-plasma products protect against infection in B19-seronegative recipients when only low levels of B19 DNA (i.e., $<10^{3.5}$ GE/mL) are present. Finally, we have shown that, in the presence of $1.6–2.2 \times 10^6$ IU of B19 DNA/mL, B19 IgG levels of 59.5 (plasma pool PS1) and 72.0 IU/mL (plasma pool PS3), respectively, are insufficient to prevent B19 transmission to B19-seronegative recipients (group III) and subsequent seroconversion.

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

Although all recipients in group I had either lost or never developed antibody reactivity against VP1 or VP2 epitopes before transfusion, 2 of them subsequently displayed strong IgG responses against linear epitopes on the VP1-unique region only and not against VP2-D. This observation is in accordance with the work of Soderlund et al. [15] and significantly strengthens our hypothesis that VP1-specific B-cell memory is maintained only with respect to linear epitopes of the unique region of VP1, as well as with respect to VP1-N/VP2-N epitopes [12]. Recipient 01052 in group 1 exhibited the lowest increase in B19 IgG reactivity after transfusion and was seronegative for antibody reactivity against VP1-D both before and after transfusion, possibly as a result of infusion with plasma containing a B19 viral load lower than that required for reactivation of the memory response.

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

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

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

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

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