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High-Sensitivity PCR Detection of Parvovirus B19 in Plasma

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Parvovirus B19 (B19) is a human pathogen transmitted to susceptible individuals via respiratory secretions and contaminated blood or blood products. B19 levels in pooled plasma of less than 10^4 genome equivalents/ml may not be infectious, while those greater than 10^7/ml are capable of transmitting infection. A World Health Organization (WHO) B19 DNA international standard has been recently introduced. The purpose of the present work was to develop a PCR–enzyme-linked immunosorbent assay (PCR-ELISA) calibrated against the WHO B19 DNA international standard which could easily and reliably detect B19 DNA levels in plasma above 10^4 IU/ml (6.5×10^3 genome equivalents/ml). A B19 PCR-ELISA system was developed which uses a dinitrophenylated oligonucleotide probe to detect immobilized biotinylated amplicons following single-round PCR amplification. The level of B19 DNA (in international units per milliliter) in individual and pooled plasma specimens was evaluated. Proteinase K treatment of plasma was found to be sufficient to quantitatively release B19 DNA. The B19 PCR-ELISA had a sensitivity of detection of 1.6×10^3 IU/ml B19 DNA and a dynamic range extending from 8 to 1,000 IU of B19 DNA (equivalent to 1.6×10^3 to 2×10^5 IU of B19 DNA/ml). Furthermore, the antibody profile of pooled plasma products was determined in terms of B19 immunoglobulin G (IgG) (in international units per milliliter). The B19 IgG level was found to be 64.7 ± 17.5 IU/ml (mean ± standard deviation). The B19 PCR-ELISA, which is calibrated against the B19 DNA international standard, may have an application for the rapid screening of plasma minipools for B19 DNA, thereby leading to an improvement in blood product safety.

Parvovirus B19 (B19) is the causative agent of multiple human diseases, including extensive fetal loss and severe disease or even death in immunocompromised individuals (20, 22). Indeed, it can be estimated that upwards of 3,000 pregnancies per annum may be lost in the European Union and the United States due to B19 infection, based on an infection rate of 0.1% and a susceptible cohort of over 3 million pregnancies involving seronegative females. In Europe, it has been proposed that 15% of immunocompromised patients may succumb to B19 infection per annum, leading to a resultant mortality rate of 7% in this patient cohort (20).

While viral infection can occur though either respiratory secretions or contaminated blood or blood products, the precise viral load required to initiate infection is unknown. Consequently, B19 contamination of pooled blood products is of major significance, primarily due to the high physicochemical tolerance of B19 to many of the treatments used in plasma processing allied to the extremely high levels of viremia in acutely infected, and often asymptomatic, individuals ($>10^{12}$ genome equivalents/ml) (15). These factors combine to present significant challenges to manufacturers in terms of the production of B19-free material. Indeed, in the absence of official regulations to control the safety of blood or blood products with respect to B19 presence, individual manufacturers have established minipool screening protocols to minimize B19 contamination (1).

While serological diagnosis of viral infection is now well standardized and widely available (6, 12), only a limited number of methods for the extraction and detection of B19 nucleic acid in single or pooled blood products have, as yet, been described (1, 3, 11, 19, 24). These assays primarily utilize either digoxigenin (DIG)-labeled UTP incorporation into newly synthesized amplicons during PCR to facilitate detection or quantitative PCR technology such as Taqman chemistry (1, 24). Despite numerous advantages, application of these systems to B19 DNA detection in either individual or pooled plasma units may be limited by irreproducibility of DIG-UTP incorporation, low sensitivity, and cost factors.

Nonetheless, the availability of these methods—combined with reliable methods for antibody detection and the introduction of international-standard preparations for the quantitation of B19 immunoglobulin G (IgG) and, more recently, B19 DNA—is leading to further improvements in the situation in which manufacturers had depended on the presence of high levels of B19 IgG alone, in pooled plasma products, as an indicator of product safety (9, 13, 15, 17). Furthermore, a recent report has shown that 1 IU of B19 DNA equals 0.65 genome equivalent (1). Despite these advances, a recent study has highlighted problems with both specimen preparation and lack of test system standardization for the detection of B19 DNA (17). Moreover, it has been shown that B19 can persist, at low levels, in immunocompetent individuals for up to 6 to 40 months postinfection (2, 14), implying that ultrasensitive B19 DNA detection is not be suitable for plasma screening due to detection of viremic, though noninfectious, plasma units. Furthermore, the recent observation that preparations of a pooled plasma product (Solvent/Detergent Treated Viplas/SD), containing less than 10^4 genome equivalents/ml, did not lead to seroconversion in healthy, B19-seronegative volunteers while under evaluation in a phase IV clinical trial has led to the consensus that this level of B19 contamination may be a safe

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TABLE 1. Nucleotide sequences of oligonucleotide primers and their relative positions in the B19 genome[a]

Primer or probe	Gene	Position	Sequence (5′ → 3′)
Primers			
F1	NS1	1399–1422	Biotin-AATACACTGTGGTTTTATGGGCCG
R1	NS1	1600–1576	CTAAAATGGCTTTTGCAGCTTCTAC
Probe (DNP-Oligo)	NS1	1536–1566	$(DNP)_3$-CTTAATAATACCTTCATCCCAGACCACCAA

[a] The oligonucleotide primers were selected based on previous observations (5, 7). The relative nucleotide positions of the B19 primers are numbered according to reference 21. Primer F1 was biotinylated at the 5′ end to facilitate binding of amplicons to streptavidin-coated microtiter wells. The detection probe was labeled with three DNP groups at the 5′ end to facilitate IgG (anti-DNP)-horseradish peroxidase conjugate binding.

viral load which is incapable of transmitting B19 infection to blood product recipients (4).

Here, we report the development of a robust and reliable microplate detection system for the detection of biotinylated PCR product using dinitrophenyl (DNP)-labeled probes, in combination with antibody-enzyme conjugate, for the detection of B19 DNA in serum or plasma at a sensitivity of 1.6×10^3 IU/ml, which may have a utility in plasma screening.

MATERIALS AND METHODS

Specimens. Aliquots of Solvent/Detergent Treated Viplas/SD plasma units (PS1 to -32), each comprising 5,000 individual donations, were obtained from Aristides Lazo (VI Technologies, Boston, Mass.) while individual units of parvovirus B19-containing plasma (IDS1 to -7) were kind gifts from both Albrecht Groener and Thomas Weimer (Aventis Behring, Marburg, Germany). The World Health Organization (WHO) B19 DNA international standard (99/800) for nucleic acid testing was obtained from the National Institute of Biological Standards and Control, Potters Bar, Hertfordshire, United Kingdom (18a). This material, which contains intact B19 virions, consists of lyophilized, pooled plasma containing a known copy number of 5×10^5 IU of B19 DNA/vial. All other specimens which were used to confirm PCR–enzyme-linked immunosorbent assay (PCR-ELISA) specificity ($n = 200$) were obtained from the Irish Blood Transfusion Service (Dublin, Ireland). A control plasmid (pB19NS1) containing the entire B19 NS1 coding region was used as positive control for all PCRs and was prepared as previously described (8). DNA concentration of pB19NS1 was computed from A_{260} measurements and was used in parallel with the B19 DNA international standard in the establishment of test sensitivity. Note: B19 levels are expressed in various ways throughout this report, which reflects how levels were reported in the original publications cited.

Specimen preparation and B19 DNA amplification. Undiluted or diluted (10^{-4} to 10^{-10} in phosphate-buffered saline) specimens (100 μl) were digested with 2 μl of proteinase K (20 mg/ml) at 56°C for 1 h, and this was followed by boiling and centrifugation (13,000 × *g* for 30 min at 4°C) to remove precipitated protein. Specimens were also extracted with the QiAmp Blood kit (QIAGEN, Hilden, Germany) as described by the manufacturer (where indicated). DNA extraction was performed in a laboratory separate from that in which PCR reagent preparation and nucleic acid amplification were carried out. Template DNA present in the supernatant (5 μl) was added to the following mixture: 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1%(vol/vol) Triton X-100, 2.0 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate (Promega, Madison, Wis.), 1 M betaine (which enhanced the PCR) (Sigma, Poole, United Kingdom) (data not shown), a 1.0 μM concentration of each oligonucleotide primer (Table 1) (5, 7) specific for regions within the NS1 coding region of the B19 genome, and 1.25 U of *Taq* polymerase (Promega) in a total volume of 50 μl. Amplification was as follows: 95°C for 6 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, terminating in 72°C for 5 min. Ten microliters of each PCR product was subjected to 1% (wt/vol) agarose gel electrophoresis with ethidium bromide (0.5 μg/ml; Sigma) for 30 min at 100 V. Amplicon visualization was performed using an Eagle-Eye II gel documentation system (Stratagene, La Jolla, Calif.).

Microplate preparation and immunoassay format. Microwells (Nunc, Roskilde, Denmark) were coated with streptavidin (2.5 μg/ml) and stabilized for 4°C storage by the addition of 1% (wt/vol) bovine serum albumin in 50 mM sodium carbonate buffer, pH 9.4. Biotinylated PCR products were diluted 1/20 in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to give a final volume of 100 μl, added to microwells coated with streptavidin, and incubated at 37°C for 30 min. After two washes with phosphate-buffered saline–0.05% (vol/vol) Tween 20 (PBST), 100 μl of 125 mM NaOH–100 mM NaCl was added to the microwells to dissociate DNA duplex, incubated at room temperature for 10 min, and washed four times with PBST. Then, 100 μl of DNP-labeled oligonucleotide (Table 1) (100 ng/ml in 6× SSC–0.1% [wt/vol] SDS) was added to the microwells and incubated for 1 h at 60°C followed by four washes with PBST. Microwells were then blocked with 2.5% (wt/vol) milk powder in PBST for 1 h at 20°C. Following removal of the blocking solution, IgG (anti-DNP)-horseradish peroxidase conjugate was added, and the mixture was incubated at room temperature for 30 min and washed four times with PBST. Substrate (100 μl of tetramethylbenzidine) was then added, and the mixture was incubated for 15 min at room temperature. The reaction was terminated by the addition of 1 N sulfuric acid and measured spectrophotometrically at 450 and 630 nm (Dynatech MRX).

Serology. B19 IgG and IgM immunoassays were obtained from Biotrin (Dublin, Ireland) and were used as previously described (12) and in accordance with manufacturers' instructions. Briefly, all immunoassays utilized conformationally intact B19 capsid antigens (VP1 and VP2) for antibody capture, except the Western blot immunoassay for B19 IgG, which contains denatured B19 capsid proteins (12).

RESULTS

Assay sensitivity. PCR-ELISA sensitivity of detection was evaluated using dilutions of both the WHO B19 DNA international standard (99/800) and purified control plasmid (pB19NS1) containing the entire NS1 gene. ELISA analysis of the B19 DNA international standard, following proteinase K extraction and PCR amplification using primers F1 and R1, facilitated detection of 8 IU of B19 DNA/5 μl (1.6×10^3 IU/ml) (Fig. 1). ELISA evaluation, following extraction using the Qiagen spin column, resulted in the detection of 40 IU (8×10^3 IU/ml) of the B19 DNA international standard (Fig. 1). These results indicated that proteinase K digestion of the international standard resulted in superior detection relative to that of the spin column-extracted material, and proteinase K digestion was thus used in subsequent experiments. Figure 1 illustrates a calibration curve, extending over 3 orders of magnitude, generated following PCR amplification of half-log dilutions of the B19 DNA international standard which was subsequently used to compute B19 DNA levels (in international units per milliliter) in individual and pooled plasma specimens. Furthermore, the improvement in PCR-ELISA sensitivity (8 IU; 1.6×10^3 IU/ml) over that obtained with ethidium bromide staining (200 IU; 4×10^4 IU/ml) following agarose gel electrophoresis is also evident (Fig. 1).

A sensitivity of detection of 50 copies of the control plasmid (in 5 μl) was determined, which equates to 10^4 copies/ml. Similarly to the WHO international standard, the sensitivity of detection by agarose gel electrophoresis was observed to be 10 times lower (500 copies of control plasmid [10^5 copies/ml]). Thus, the ELISA format confers at least a 10-fold increase in sensitivity over ethidium bromide visualization of amplicons.

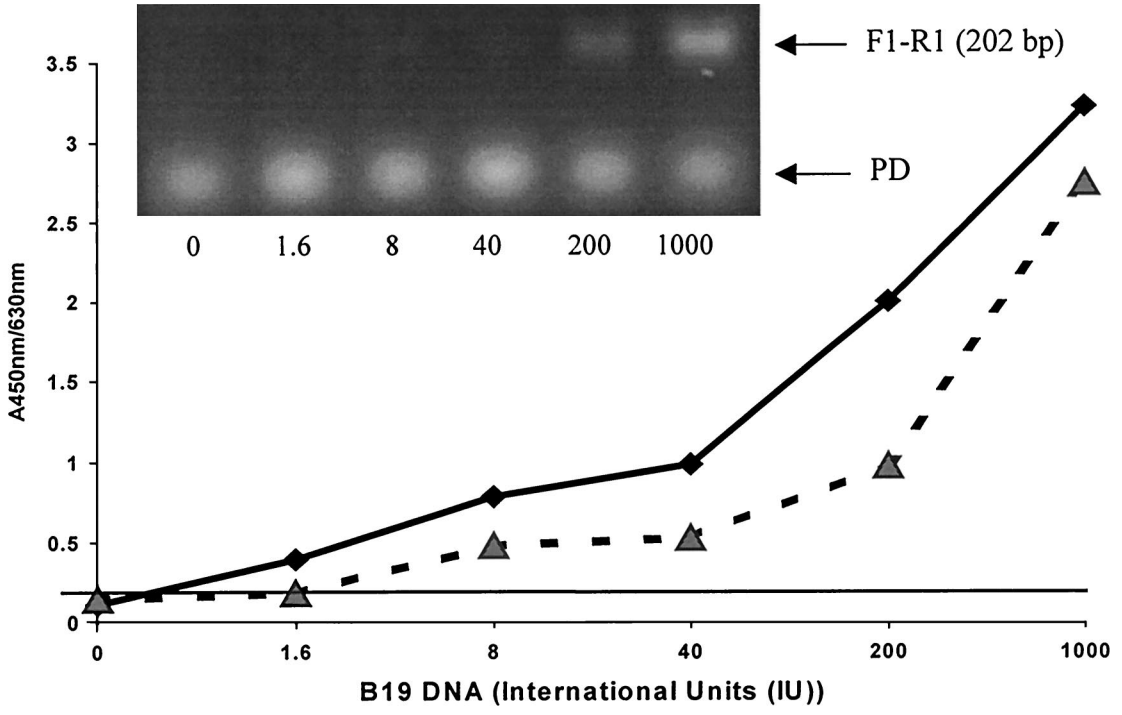

FIG. 1. PCR-ELISA quantitation using the WHO B19 DNA international standard. Half-log dilutions were extracted by proteinase K (solid line) or spin column (dashed line) methodologies, subjected to PCR using primers F1 and R1, and analyzed by ELISA. Absorbance at 450 and 630 nm is plotted versus the log amount of B19 DNA. The PCR-ELISA cutoff (0.159) is indicated by the horizontal black line and was calculated as the mean plus 2 standard deviations [0.107 + 2(0.026)] following replicate analysis of 20 B19-DNA-negative specimens. A level of 8 IU of B19 DNA was reliably detectable following proteinase K extraction. Amplicon detection by agarose gel electrophoresis detection is also shown (inset). It can be seen that a minimum of 200 IU of B19 DNA only can be detected by this approach. PD, primer dimer.

Specimen evaluation. PCR-ELISA analysis of pooled plasma specimens (PS1 to -30) detected high levels of viremia in specimens PS1 to -5 (Fig. 2). All other pooled plasma specimens tested contained either low levels of B19 DNA or levels which were below the sensitivity of the test system. Subsequently, the levels of B19 DNA present in specimens PS1 to -5 were quantified and found to contain between 8.0×10^7 and 5.0×10^8 IU of B19 DNA/ml, in addition to B19 IgG levels of 59.5 to 86.5 IU/ml, which were significantly higher than the immunoassay cutoff (3 IU/ml) (Table 2). No B19 IgM was detectable in these pools (Table 2) or any other pooled plasma specimen. All specimens tested were positive for B19 IgG in all commercial immunoassay systems, including microplate enzyme immunoassay and immunofluorescent, Western, and immunoblot assays. No specimen reactivity was observed against denatured VP2 when tested by Western blotting; however, all specimens were VP1 positive by this method. The B19 IgG level (mean ± standard deviation) in all plasma pools tested was found to be 64.7 ± 17.5 IU/ml ($n = 30$).

Higher levels of B19 DNA (1.2×10^9 to 8.0×10^{11} IU/ml) were found following evaluation of individual plasma donations (IDS1 to -7). Significantly, all of these specimens were B19 IgG negative, and only one contained detectable B19 IgM, suggesting that all individuals were recently infected with B19 (Table 2). Finally, a pool of 200 serum specimens also tested negative by the PCR-ELISA, further confirming assay specificity (specimen/cutoff absorbance ratio, <1.0).

DISCUSSION

A robust microwell detection system for the high-sensitivity detection of biotinylated amplicons following extraction and single-round PCR amplification of parvovirus B19 DNA has been developed. The overall amplification and detection system uses colorimetric detection for specimen visualization, exhibits a sensitivity of detection of 1.6×10^3 IU of B19 DNA/ml, is compatible for use with either individual or pooled specimens, and is capable of being automated. Significantly, the requirement for PCR-dependent incorporation of a detection moiety (e.g., DIG) is obviated.

Although the use of commercial extraction kits is thought to reduce the presence of PCR inhibitors, especially for the determination of viral load in plasma pools (18), the data presented here demonstrate that proteinase K digestion alone is sufficient to quantitatively release B19 DNA from pooled and individual plasma specimens treated with citrate. Extraction of specimen DNA is of paramount importance to ensure quantitative release of target DNA, and a number of methods for DNA extraction based on efficiency, convenience, reproducibility, and the presence of inhibitors have been investigated (25). These authors found that treatment of serum specimens by rapid heating fulfilled the criteria for routine diagnosis while other extraction methods, such as lysis treatment and commercial DNA extraction kits, failed to yield higher levels of extracted DNA based on quantitative PCR results. Our observa-

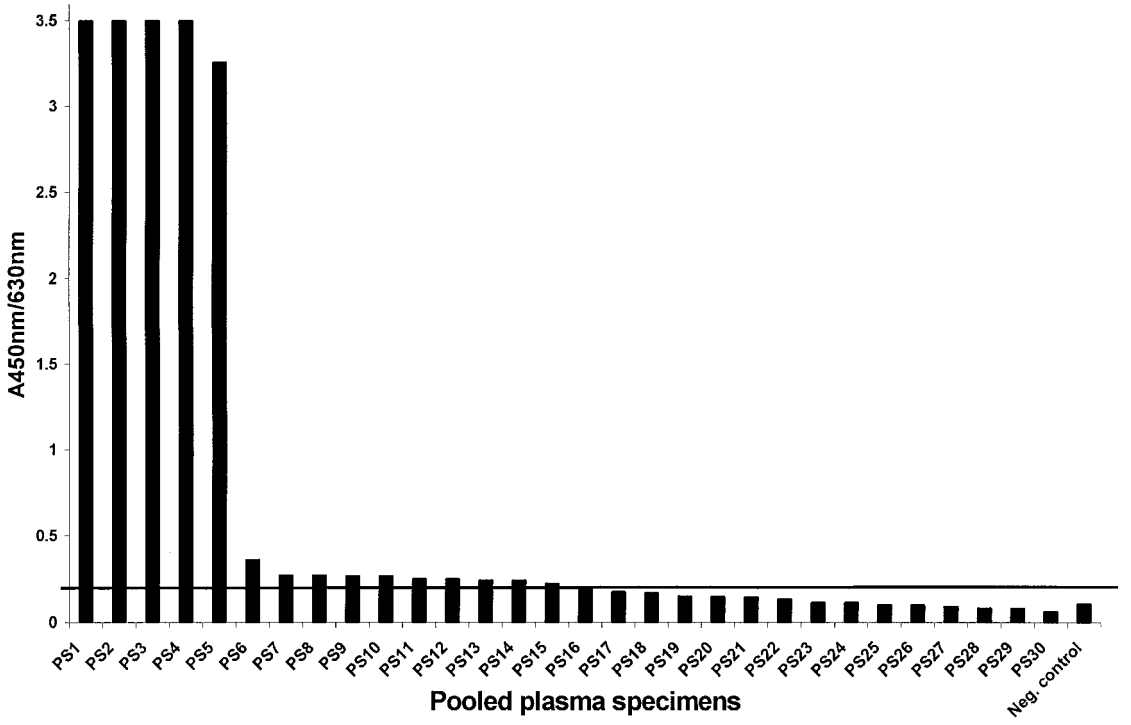

FIG. 2. Qualitative detection of B19 DNA in 30 pooled plasma specimens by PCR-ELISA. Five pools (PS1 to PS5) were found to have high levels of B19 DNA, the levels of which were subsequently quantified in terms of B19 DNA (in international units per milliliter) (see text). Eleven pools (PS6 to PS15) appeared to contain low levels of B19 DNA. In 14 pools (PS17 to PS30), any B19 DNA present was below the PCR-ELISA cutoff of 1.6×10^3 IU/ml.

tions for citrate-treated plasma are in agreement with those found by Zerbini et al. (25), who found that specimen treatment with proteinase K was more efficient for B19 DNA isolation than commercial kits. This observation should be of significant utility in the large-scale screening of plasma by blood product manufacturers.

A sensitivity limit of 350 to 3,500 copies of B19 DNA has been reported (7) when single-round PCR with optimized primer pairs and ethidium bromide detection has been used following agarose gel electrophoresis. Using these primers, in combination with a modified PCR procedure and ELISA-based amplicon revelation, a minimum of 8 IU of B19 DNA was detectable in the present study, which represents a significant improvement over this previous work. Although Durigon et al. (7) showed that nested PCR improved sensitivity by up to 100-fold, this option is not entirely suitable for large-scale plasma screening, due to contamination risks.

TABLE 2. B19 virological and serological status of pooled plasma and individual specimens

Specimen[a]	Level in plasma		
	B19 DNA (IU/ml)	B19 IgG (IU/ml)	B19 IgM (index[b])
PS1	2.2×10^8	59.5	0.19
PS2	5.0×10^8	74.0	0.22
PS3	1.6×10^8	72.0	0.23
PS4	1.2×10^8	86.5	0.23
PS5	8.0×10^7	84.0	0.21
IDS1	8.0×10^{11}	0.0	0.13
IDS2	7.0×10^{11}	0.0	0.08
IDS3	2.0×10^{11}	0.0	0.08
IDS4	1.4×10^{11}	0.0	0.26
IDS5	1.2×10^9	0.0	2.02
IDS6	1.1×10^{11}	0.0	0.24
IDS7	3.2×10^{11}	0.0	0.60

[a] PS1 to −5, pooled plasma specimens; IDS1 to −7, individual specimens.
[b] B19 IgM values are expressed as index values (ratio of specimen optical density to cutoff value optical density). An index of >1.1 implies B19 IgM positivity.

In the last decade a number of quantitative PCR assays have been developed to detect B19 DNA in individual serum and plasma specimens; however, few have directly addressed either the issue of plasma pool contamination or assay compatibility with the recently introduced WHO B19 DNA international standard. Furthermore, these assays may exhibit inappropriate sensitivity for pooled plasma screening. For instance, Zerbini et al. (24) reported reliable detection of 60 B19 genome equivalents (1.2×10^4 /ml) using DIG-labeled PCR product detection, which is above the B19 level (10^4 genome equivalents/ml) thought to represent a noninfectious dose. Conversely, other authors have reported assay sensitivities of 168 and 100 B19 copies/ml, respectively, the use of which may lead to discarding viremic, though noninfectious, plasma units (10, 19). Although dot blot hybridization assays using colorimetric substrates (EnVison) have been developed (26) which can detect 3×10^3 copies (10 fg) of B19 DNA (while the addition of chemiluminescent substrates increased the sensitivity to 6×10^2 genome copies [2 fg]), these test formats may not be readily automated for large-scale specimen processing.

Whole-virus detection using receptor-mediated hemagglutin-

nation has been proposed as a strategy for the detection of B19 in plasma (16, 23). However, due to documented false negativity in the presence of B19 IgG and IgM and the absence of precise sensitivity of detection in terms of genome equivalents per milliliter (23), this method does not sufficiently guarantee blood product safety, in terms of the absence B19 contamination. The mean plasma B19 IgG level of 75.2 IU/ml observed in pooled plasma specimens PS1 to -5 strongly suggests that antibody presence in plasma does not interfere with B19 DNA extraction or, conversely, that B19 presence in plasma pools does not interfere with B19 antibody detection in serological assays. Since the individual donations screened were all B19 IgG negative, it is not possible to predict if free virus interferes with antibody detection in specimens obtained from individual donors. However, it should be noted that specimen IDS5, which was also B19 IgM positive, contained 100 times less B19 DNA than all other individual specimens tested. This observation may reflect virus diminution as the humoral response is mounted. The presence and absence of IgG reactivity against linear epitopes of VP1 and VP2, respectively, are consistent with results obtained following the serological evaluation of individual serum samples by Western blot analysis (12). These results suggest that the potential protective nature of B19 IgG present in plasma pools primarily comprises antibody reactivity directed against both conformational and VP1-unique region epitopes on B19 antigens.

The introduction of a WHO B19 DNA international standard represents a significant advance in the improvement of blood product safety and will serve to diminish ambiguity between results from different organizations currently using either various assay formats or quantitative terminology to express B19 DNA levels (i.e., genome equivalents per milliliter, copies per milliliter, and PCR-detectable units per milliliter [17]). Although there is no definitive information regarding the precise B19 viral load in plasma which can transmit infection to recipients, it has been reported that levels greater than 10^7 genome equivalents/ml, in three individual lots of pooled plasma (Solvent/Detergent Treated Viplas/SD), resulted in B19 seroconversion in 19 recipients (4). Consequently, there is now an impetus to identify and remove individual high-titer B19 plasma donations from plasma pools, and a number of companies have introduced minipool screening to address this problem.

In conclusion, the currently recommended maximum level of 10^4 genome equivalents/ml of B19 DNA in plasma pools as a safe viral load suggests that the B19 PCR-ELISA presented here, which is calibrated against the international standard and has a cutoff of 8 IU of B19 DNA (1.6×10^3 IU of B19 DNA/ml), has an application for the rapid screening of plasma donations or minipools for parvovirus B19 DNA, thereby leading to an improvement in blood product safety.

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