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Improved detection of acute parvovirus B19 infection by immunoglobulin M EIA in combination with a novel antigen EIA

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Vox Sanguinis

Background and Objectives Although parvovirus B19 is a significant blood product contaminant, few methods other than polymerase chain reaction (PCR) have been developed to detect the presence of the virus.

Material and Methods A B19 antigen enzyme immunoassay (EIA) has been developed and the sensitivity of detection is ascertained using dilutions of the B19 capsid protein VP2 and 10-fold dilutions of B19 viraemic serum. Once the assay cut-off was established, a panel of viraemic donations (n = 70) was screened by the antigen EIA. The B19 immunoglobulin M (IgM) and IgG status of these specimens was also determined. During screening of blood donor units by quantitative PCR, 70 individuals were identified with levels of B19 DNA greater than 10^6 IU/ml at the time of blood donation.

Results The sensitivity of the B19 antigen EIA was estimated to be equivalent to between 10^8 and 10^9 IU/ml B19 DNA or 1–10 pg/ml of recombinant capsid protein. B19 detection was significantly enhanced when viraemic specimens were pretreated with a low pH proprietary reagent. Unlike other virus-detection assays, detection of the B19 antigen was not affected by the presence of B19 IgM or IgG antibodies. In addition, the assay was capable of detecting all three genotypes of human erythrovirus. Combined specimen analysis by the B19 antigen assay and a B19 IgM assay facilitated the detection of 91% of acute B19 infections in the test population.

Conclusion In combination with B19 IgM detection, application of the B19 antigen EIA is a flexible and efficient method of detecting recent B19 infection and can be used as an alternative to PCR.

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Key words: antigen EIA, B19 IgM, blood products, erythrovirus.

Introduction

Parvovirus B19 (B19V) infection of immunocompromised patients may result in severe morbidity and mortality [1,2]. Moreover, B19 infection of pregnant women may lead to fetal death [3]. The recent implementation of minipool polymerase chain reaction (PCR) screening procedures for pooled plasma, combined with mandatory European guidelines on acceptable B19 contamination of human immunoglobulin preparations (< 10 000 IU/ml B19 DNA), will minimize B19 contamination and improve the safety of pooled blood products [4,5]. However, the extremely high levels of B19 viraemia in recently infected individuals (10^{13} IU B19 DNA/ml) [6], asymptomatic B19 infections and the resilience of the virus to many of the virus-inactivation procedures mean that

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B19 screening and elimination are still problematic [7,8]. Although PCR is currently the method of choice, contamination issues during screening [9], accurate erythrovirus genotype detection [10] and lack of individual donation screening necessitate continual evaluation of emerging technologies to ensure blood product safety.

Currently, B19 viral capsid protein production *in vivo* is detected by immunofluorescent staining and receptor-mediated haemagglutination (RHA) assays whereas viral DNA production is detected by PCR, dot blot hybridization and quantitative PCR (qPCR) [11–15]. RHA does not detect the B19 antigen at the required sensitivity in specimens that contain B19 IgG/M [11,15]. This is unacceptable especially when B19 IgG positive solvent/detergent-treated plasma, contaminated with B19 DNA, has been shown to transmit infection [16].

B19 antigen detection by enzyme immunoassay (EIA) is an alternative strategy for individual donor screening but may also be confounded by low assay sensitivity, differential reactivity between VP2 capsid and native B19 antigen detection and B19 antibody presence [17,18]. The B19 antigen assay described by Lowin *et al.* [18] has an apparent sensitivity of detection for recombinant VP2 capsids of 10^8 particles per ml; however, application of the assay to native B19 antigen detection was not demonstrated.

Using a Food and Drug Administration (FDA)-cleared B19 IgM EIA [19], Beersma *et al.* [20] have shown that in sera with B19 DNA levels greater than 10^6 per ml, B19 IgM reactivity always exceeds 3·0 (EIA cut-off = 1·0). Thus, it is clear that the presence of B19 VP2 IgM antibodies in sera is predictive for the presence of B19 DNA. This observation represents the first data unambiguously correlating B19 viral load with IgM antibody levels. Importantly, it also provides for an alternative strategy, employing simultaneous B19 IgM and antigen detection, to overcome the sensitivity issues pertaining to B19 antigen detection in individual donor units. Here, we show that such a strategy facilitates detection of B19 antigen levels in plasma donations.

Materials and methods

B19 antigen EIA optimization

Recombinant B19 VP2 capsids were expressed and purified as previously described [21] and were used for sheep and rabbit immunization. Affinity purified sheep IgG (anti-B19 VP2) was coated onto microtitre plates (Nunc Maxisorp, Roskilde, Denmark) and the rabbit IgG (anti-B19 VP2) was conjugated to horseradish peroxidase (HRP), as described by Hermanson [22], and was used to detect captured B19 antigen.

Optimal IgG (anti-B19 VP2) plate-coating concentration (4 μg/ml) and conjugate dilution (1/4000 dilution) were established by testing B19-viraemic and non-viraemic plasma specimens. Dilutions of B19 VP2 capsids from 0·01 to 10 000 ng/ml were also analysed by the antigen EIA to determine the limit of detection in terms of protein concentration. The mean absorbance of the negative control for each batch of VP2 plus three standard deviations was used to set the assay cut-off value (COV).

To determine sensitivity in terms of B19 viral antigen detection, viraemic plasma was evaluated (qPCR testing was performed at the National Genetics Institute, CA, USA and results were reported in copies/ml). The mean absorbance of a panel of 201 non-viraemic human plasma samples plus three standard deviations was used to set the assay COV. This was matched to a dilution of a B19-viraemic plasma, which was used in all subsequent assays as a cut-off calibrator and facilitated determination of the positive or negative status of specimens tested on the antigen EIA.

Specimen preparation and final assay procedure

Test plasma and control specimens were diluted (1/5) in a low pH proprietary diluent (citrate buffer-containing detergents; available from Biotrin International Ltd., Dublin, Ireland) and were added to IgG (anti-B19 VP2) sensitized microwells (100 μl per well) for 1 h. Following a wash step, the rabbit IgG (anti-B19 VP2)–HRP conjugate was incubated in the wells for 30 min. Tetramethylbenzidine substrate (BioFX Laboratories Inc., Owings Mills, MD, USA) was added to the wells for 30 min. The reaction was terminated using 1 N sulphuric acid and the absorbance was measured at 450/630 nm. The presence of B19 antigen in a sample was determined by the absorbance ratio of specimen sample to cut-off calibrator sample (index value; IV). Specimens yielding index values ≥ 1·0 were classed positive while those < 1·0 were deemed negative.

Parvovirus B19 IgM and IgG

All specimens in this study were screened for B19 IgM and B19 IgG using commercial assays (Biotrin) as described previously [21].

Donor screening by B19 qPCR

The blood donor population in The Netherlands was screened for B19V over an 18-month period (February 2003 –July 2004) using qPCR analysis as described previously [12]. Test pools of 480 were made from smaller pools of 48 donations. A pool identified with > 10^4 IU/ml B19 DNA was resolved via test pools of 48 donations and subsequently eight donations to trace the viraemic donor(s). Identified viraemic donations (n = 70) were then used to evaluate the B19 antigen EIA [12]. Results were expressed in IU/ml [23]. The copies-to-IU conversion factor has been calculated previously to be 3·34 [14].

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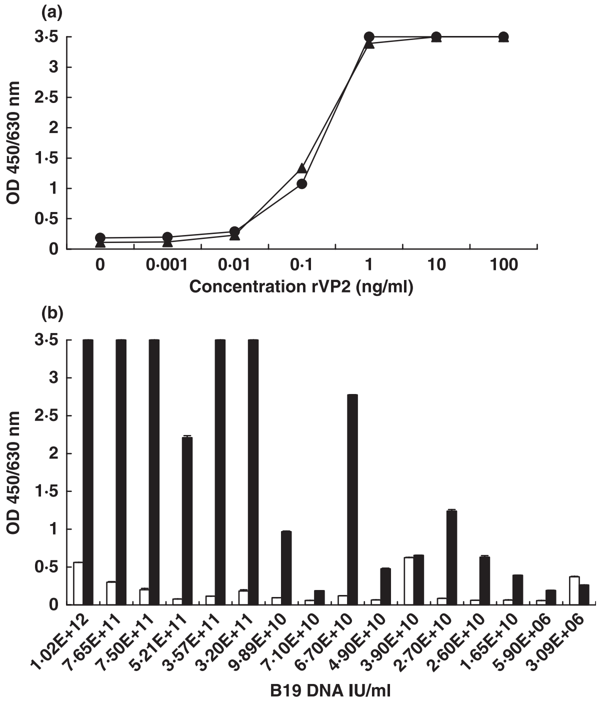

Fig. 1 Determination of B19 antigen enzyme immunoassay (EIA) assay sensitivity. (a) Two independent batches of recombinant capsid VP2 (rVP2), V056 (circles) and V057 (triangles) were decimally diluted to determine assay sensitivity. (b) Comparison of specimen diluents used in the detection of B19 viral capsids. Specimens were diluted in either Tris-buffered saline Tween-20 (TBST) (clear boxes) or a low pH proprietary reagent (filled boxes). Error bars represent the standard deviation from the mean.

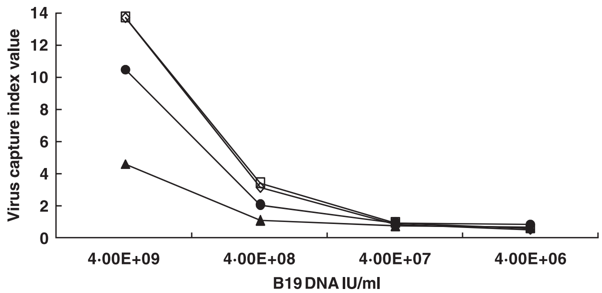

Fig. 2 Determination of antigen assay sensitivity using titrations of polymerase chain reaction (PCR)-quantified viraemic specimens. Viraemic plasma Bt72 (diamonds), Bt73 (squares), Bt80 (triangles) and genotype 2 Bt81 (circles) were decimally diluted in B19 negative serum to determine assay cut-off.

Results

Assay optimization and validation

Figure 1a shows identical standard curves [absorbance $_{450/630\ \text{nm}}$ vs. B19 recombinant VP2 capsid concentration (ng/ml)] generated from two independent batches of recombinant VP2 capsids in the B19 antigen EIA. These standard curves show that the minimal detectable level of B19 VP2 capsid detectable was 0·01 ng/ml, which theoretically equates to $1{\cdot}9 \times 10^6$ viral particles per ml.

However, detection of B19 viraemic plasma in the same assay format required the implementation of an alternative specimen diluent (Fig. 1b). Here, dilution of viraemic specimens (n = 16) in a low pH, proprietary diluent, compared to using Tris-buffered saline Tween-20 (TBST), facilitated a considerable increase in virus capture in the majority of specimens (0- to 30-fold). Only one specimen ($3{\cdot}9 \times 10^{10}$ IU/ml B19 DNA) that was negative for B19 IgM did not display a significant signal increase post-treatment, but did remain positive. Interestingly, the two specimens with the highest absorbance values in the assay without low pH pretreatment were IgM negative. Non-viraemic plasma remained unreactive when subjected to the same pretreatment (data not shown). Assay specificity was determined by screening non-viraemic plasma (n = 20), all of which were unreactive in the antigen EIA based on the cut-off calibrator sample (data not shown).

The assay sensitivity (limit of detection) was estimated using dilutions of viraemic specimens and was shown to be approximately between 4×10^7 and 4×10^8 copies per ml B19 DNA (Fig. 2). However, the cut-off calibrator used in the EIA contained 10^9 copies per ml B19 DNA as determined by qPCR, which equates to 2×10^7 copies B19 DNA per microwell. To further define the limit of detection, plasma specimens (n = 17), containing a range of B19 DNA concentrations and B19 IgM/G reactivity, were subsequently screened in the antigen EIA. Table 1 shows that 53% (9/17) of specimens, all of which contained greater than $1{\cdot}4 \times 10^{11}$ copies per ml B19 DNA, were also detectable in the antigen EIA. One specimen containing $7{\cdot}2 \times 10^8$ copies per ml B19 DNA, which was B19 IgM reactive, tested borderline positive (IV = 1·0) in the antigen EIA. All remaining specimens, which contained less than $1{\cdot}9 \times 10^7$ copies per ml B19 DNA and either B19 IgM or IgG or both, were unreactive in the antigen EIA.

Detection of the B19 antigen in the presence of specimen-derived B19-specific IgG or IgM is essential to avoid false negativity. Table 2 clearly illustrates that specimen-derived B19 antigen is detectable in the presence of both B19 IgG and IgM (n = 8), IgM only (n = 2) or IgG only (n = 3). Furthermore, B19 antigen is also detectable in specimens Bt72 and Bt73, which contained B19 IgM (Fig. 2). It is clear, therefore, that only B19 levels greater than 4×10^7 B19 DNA copies per ml are detectable in the antigen EIA and that the presence or absence of IgM or IgG in the specimen does not affect detection of the B19 antigen (Fig. 2 and Table 2). A specimen containing erythrovirus genotype 2 (specimen Bt81) was detected as well as erythrovirus genotype 1 (specimens Bt72, Bt73 and Bt80) in the antigen EIA (Fig. 2). Furthermore, erythrovirus genotype

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Table 1 Parvovirus B19 detection by antigen enzyme immunoassay (EIA) and serological analysis (B19 IgM and IgG) of specimens previously quantified by polymerase chain reaction (PCR) (copies per ml). For the antigen EIA an index value (IV) ≥ 1·0 is positive (+) and < 1·0 is deemed negative (–). For both the B19 IgM and IgG EIA IV > 1·1 is positive; IV < 0·9 is negative; and IV between < 1·1 and IV > 0·9 is deemed equivocal (*eq*)

Sample identifier	IgM EIA	IV	IgG EIA	IV	qPCR (copies per ml)	Antigen EIA	IV
Cut-off calibrator	6·77	+	0·99	*eq*	$1{\cdot}3 \times 10^9$	1·00	+
W P	0·80	–	0·14	–	$6{\cdot}9 \times 10^{11}$	18·7	+
C4	0·26	–	0·06	–	$6{\cdot}0 \times 10^{11}$	> 3·0	+
PL19	0·59	–	0·07	–	$5{\cdot}6 \times 10^{11}$	> 3·0	+
C7	0·58	–	0·06	–	$5{\cdot}5 \times 10^{11}$	> 3·0	+
C1	0·13	–	0·04	–	$4{\cdot}8 \times 10^{11}$	> 3·0	+
C2	0·08	–	0·06	–	$4{\cdot}6 \times 10^{11}$	> 3·0	+
C6	0·24	–	0·05	–	$3{\cdot}3 \times 10^{11}$	> 3·0	+
C3	0·08	–	0·09	–	$3{\cdot}9 \times 10^{11}$	> 3·0	+
PL9	0·11	–	0·06	–	$1{\cdot}4 \times 10^{11}$	> 11·0	+
C5	2·02	+	0·17	–	$7{\cdot}2 \times 10^8$	1·0	+
E R	3·0	+	8·1	+	$1{\cdot}9 \times 10^7$	0·03	–
PL1	6·3	+	1·95	+	$1{\cdot}6 \times 10^7$	0·39	–
C8	0·15	–	2·56	+	$2{\cdot}6 \times 10^4$	0·04	–
D T	2·3	+	6·2	+	$7{\cdot}4 \times 10^3$	0·07	–
R S	6·6	+	6·8	+	$8{\cdot}9 \times 10^3$	0·42	–
PL20	0·11	–	4·78	+	550	0·42	–
PL16	0·2	–	4·80	+	200	0·39	–

Table 2 Effect of B19 IgM and IgG in plasma on the detection of B19 antigen. B19 antigen enzyme immunoassay (EIA) and serology results for plasma from patients with suspected B19 infection. For the antigen EIA an index value (IV) ≥ 1·0 is positive (+) and < 1·0 is deemed negative (–). For both the B19 IgM and IgG EIA an IV > 1·1 is positive; IV < 0·9 is negative; and IV between < 1·1 and > 0·9 is deemed equivocal (*eq*)

Sample Identifier	IgM EIA	IV	IgG EIA	IV	Antigen EIA IV
Cut-off calibrator	6·77	+	0·99	*eq*	1
931	0·14	–	0·70	–	18·6
420	0·16	–	0·90	*eq*	18·3
981	1·73	+	1·50	+	18·1
410	0·25	–	0·90	*eq*	18·1
375	0·14	–	0·70	–	18·1
939	0·30	–	0·80	–	18·0
889	4·99	+	1·70	+	17·9
976	0·17	–	1·20	+	17·8
441	3·40	+	0·80	–	17·6
973	0·28	–	1·28	+	17·3
966	1·92	+	1·46	+	17·3
936	1·21	+	1·40	+	16·3
444	0·86	–	1·00	*eq*	15·4
980	0·71	–	1·70	+	12·0
427	2·06	+	0·80	–	11·9
929	2·74	+	1·40	+	11·2
888	0·25	–	1·10	+	8·2
925	1·32	+	1·50	+	6·76
416	6·89	+	2·80	+	1·3
895	6·02	+	1·90	+	1·0

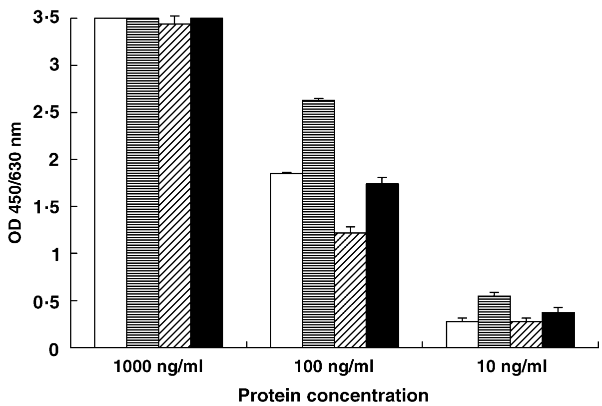

Fig. 3 Comparison of erythrovirus genotype 1 and 3 VP2 reactivity in the antigen enzyme immunoassay (EIA). Genotype 1 (clear and horizontal lined bars) and genotype 3 (diagonal lined and filled bars) recombinant VP2 was decimally diluted in either Tris-buffered saline Tween-20 (TBST) (clear and diagonal lined bars) or the proprietary low pH buffer (horizontal lined and filled bars). Error bars represent the standard deviation from the mean.

3 recombinant VP2 capsids exhibit indistinguishable reactivity in the assay to genotype 1 recombinant VP2 (Fig. 3).

Donor sample evaluation

During an 18-month period, approximately 1·4 million donations were tested for B19 DNA in The Netherlands [14], and 70 cases of asymptomatic donors (0·005%) with levels of B19 DNA greater than 10^6 IU/ml were identified. Of these, 49/70 (70%) tested positive on the antigen EIA assay for B19

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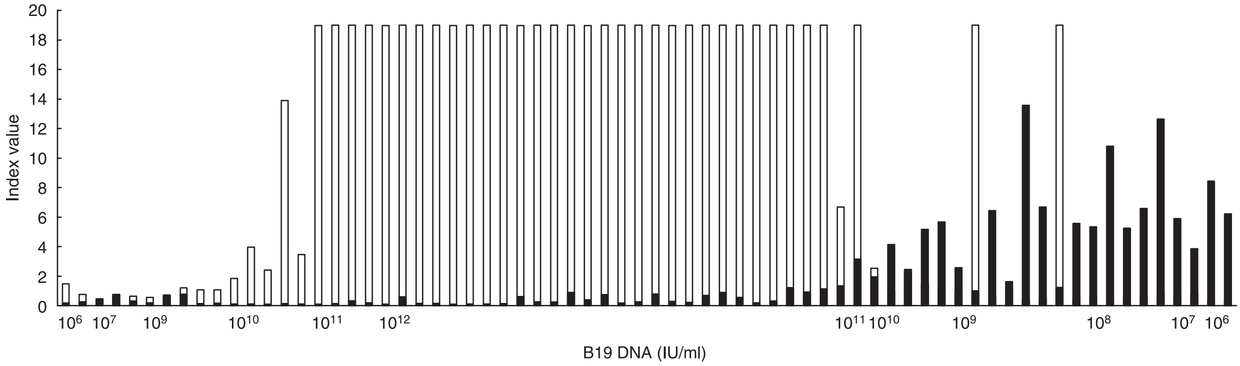

Fig. 4 A summary of the B19 antigen enzyme immunoassay (EIA) and immunoglobulin M (IgM) EIA reactivity of the panel of viraemic donors. An index value (IV) > 1·1 (denoted by line) is considered positive on both the B19 IgM EIA (filled bars) and antigen EIA (clear bars). The *y*-axis was truncated for clarity.

(range: $3{\cdot}1 \times 10^6$–$3{\cdot}2 \times 10^{12}$ IU/ml; mean: $1{\cdot}1 \times 10^{12}$ IU/ml, median: $1{\cdot}2 \times 10^{12}$ IU/ml B19 DNA) (Fig. 4). Thus, Fig. 4 depicts the combined B19 IgM and antigen EIA data of the 70 viraemic specimens, and the *x*-axis is arranged to show the rise (10^6–10^{12} IU/ml) and subsequent drop in viraemia with the development of B19-specific IgM antibodies (10^{12}–10^6 IU/ml). Testing further revealed that the panel of viraemic specimens was either pre- or early antibody seroconversion as none contained B19 IgG (data not shown).

There was a positive correlation (correlation coefficient $r = 0{\cdot}81$) between the level of B19 DNA (qPCR) and the level of B19 antigenemia (antigen EIA), but this relationship was not directly proportional. Concordance between qPCR and the antigen EIA was highest when viraemia titres were high (> 1×10^{11} IU/ml). Of the viraemic donor specimens, 27 (38·6%) tested positive (IV > 1·1) or borderline positive (two specimens were equivocal: IV ≤ 1·1, IV ≥ 0·9) for B19 IgM (Fig. 4). The specimens that were equivocal for IgM reactivity reacted strongly in the antigen EIA (IV > 19). The overlap between the two groups was considerable and 17% of the specimens tested positive for both B19 IgM and antigen (Fig. 4). Significantly, 91% of the viraemic donors were positive for either B19 IgM or antigen. Thus, these data clearly demonstrate that the combined implementation of a screening algorithm for B19 IgM and antigen readily facilitates the detection of specimens containing greater than 10^6 IU/ml B19 DNA equivalents.

Discussion

Here we describe a B19 antigen EIA for the direct detection of B19 antigen in human plasma. The detection limit of the assay was 0·01 ng/ml of purified recombinant VP2 capsids (which theoretically corresponds to $1{\cdot}9 \times 10^6$ viral particles per ml). Using dilutions of viraemic serum, the sensitivity was estimated at between 4×10^7 and 10^8 copies per ml B19 DNA equivalents. The antigen EIA was capable of detecting both erythrovirus genotypes 2 (virus) and 3 (recombinant capsids). When the antigen assay was used to test B19 viraemic donations, 70% tested positive of which had viral loads between $3{\cdot}1 \times 10^6$ and $3{\cdot}2 \times 10^{12}$ IU/ml.

B19 detection in plasma was greatly enhanced by specimen acidification. The low pH conditions may act by disrupting the viral capsid into its structural subunits, making it more accessible to the capture antibody. Although it was previously thought that B19V was highly resistant to physicochemical treatments, more recent work has shown the susceptibility of B19V to low pH treatment [24]. Boschetti *et al.* [24] showed that B19V was inactivated by greater than 5 logs after 2 h at pH 4 and that infectivity also decreased.

When the antigen assay was performed at physiological pH, the specimens that gave the highest absorbance values were B19 IgM negative, implying immune complexes hinder detection. However, when specimens were prepared in low pH conditions, neither the presence of IgM nor IgG, even at high levels, affected the detection of B19 (Table 2). It is probable that acidification caused the dissociation of any immune complexes present. False-negative results due to immunocomplexes present a problem for B19 RHA assays, which exploit the binding of a B19V receptor to red blood cells [11]. Hence, the RHA assay is ineffective for antigen detection in specimens that have seroconverted a problem resolved by the B19 antigen EIA.

B19 detection by PCR has a greater sensitivity, but such assays have many disadvantages (e.g. potential cross-contamination) not shared with an EIA. First, although erythrovirus genotypes may diverge significantly at the genomic level [25,26], requiring primer optimization [13], there does not appear to be any antigenic or immunological differences between the genotypes. The antigen EIA could identify genotype 2 erythrovirus and genotype 3 recombinant VP2 capsids at the same sensitivity as genotype 1. This is supported by the fact that all three erythrovirus genotypes can haemagglutinate human red blood cells and also infect myeloid cells with equal efficiency [27]. Second, the significance of DNA in plasma postviraemia

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is unclear as low levels of B19 DNA can persist for several years post-infection, even after IgM is lost and IgG reactivity has been established [28]. A virus detection assay, however, allows simultaneous testing of hundreds of specimens, is suitable for large-scale screening, is more economical and has a shorter time to result.

Combined B19 antigen and IgM EIA analysis of the viraemic donor specimens revealed that 91% of the donor specimens could be diagnosed as acute infection using this screening algorithm. Previously, clinical samples taken from individuals with a suspected B19 infection, which had a level of B19 DNA greater than 10^5 IU/ml, were shown to be positive for specific IgM also [20]. This was not the case with the Dutch donor specimens herein, as this panel was from asymptomatic individuals whose infection was detected due to routine screening. Donor specimens, therefore, would be from all stages post-infection including the preseroconversion stage. Experimental infection has shown that B19 infection has two phases [29], characterized by symptom-free initial high viraemia (~10^{11} copies per ml serum) followed by detectable IgM antibody and appearance of symptoms such as rash and arthralgia. IgM seroconversion causes a rapid decline of viral titre. The 70 viraemic specimens identified in this study showed a typical viraemia and IgM seroconversion pattern (Fig. 4), confirming that the donor samples are representative of all stages of acute infection.

It is important to confirm the diagnosis of acute B19 infection in a public health setting where an outbreak could lead to serious medical consequences, especially for pregnant women and immunocompromised patients. In addition, B19 screening of blood donors prior to donation would avoid the risk of contaminating blood products. The B19 antigen EIA in conjunction with specific B19 IgM detection offers an effective method of detecting acute infection.

Acknowledgements

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