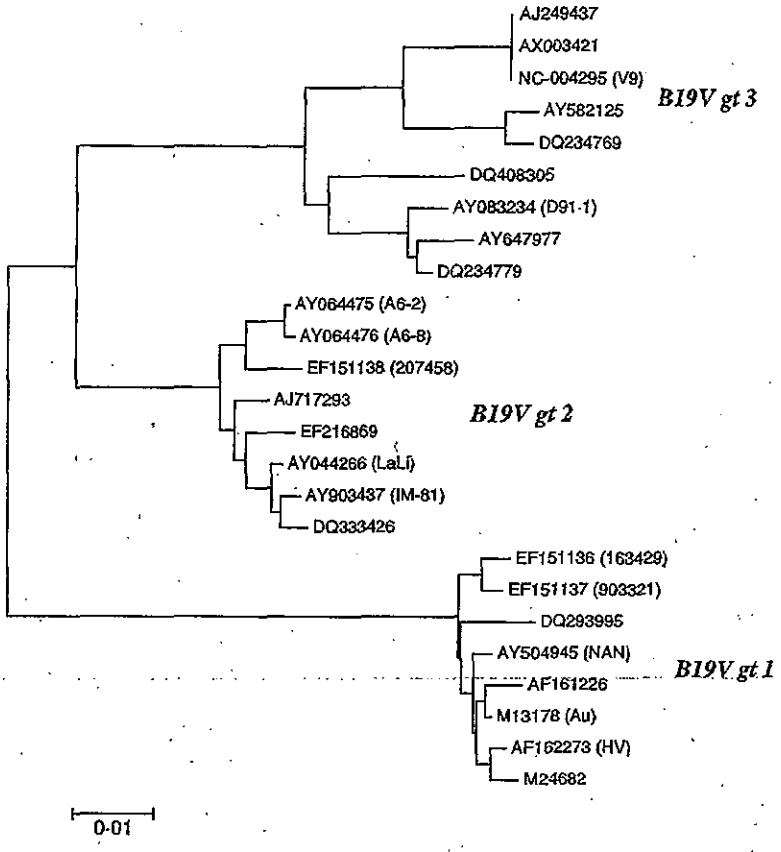

Fig. 2 Phylogenetic tree of parvovirus B19 (B19V) DNA sequences from three B19V variants (903321, 163429 and 207458) and B19V DNA sequences with published genotypes from GenBank. The sequence of a 1536-bp fragment corresponding to part of the NS1–VP1 region was used to create the tree.

(a) Roche B19V DNA assay

	FORWARD PRIMER	PROBE BINDING REGION	REVERSE PRIMER
B19V GENOTYPE 1	GGGGCAGCATGTGTTAAAGTGGATT	TACACTGTGGTTTTATGGGCCGCCAAGTACAGGAAAAACAAACTTG	ACTTTCCATTTAATGATGTAGCAGG
B19V run control	----------	----------	----------
903321	----------	----------	-T--------
163429	----------	----------	---------G---
B19V GENOTYPE 2	---T--A-----T----------	C--C---------C-----C--------T----------T---	-T---------G--
207458	---T--A-----T----------	C--C---------C-----T--------T----------T---	-T---------G--
B19V GENOTYPE 3	---T--A----------G-------	C--C---------C-----A--------T----------T---	---------G--

(b) In-house B19V DNA assay

	FORWARD PRIMER	PROBE BINDING REGION	REVERSE PRIMER
B19V GENOTYPE 1	AATGCAGATGCCCTCCAC	AACCCCGCGCTCTAGTAC	GGACCAGTTCAGGAGAATCAT
B19V run control	----------	----------	----------
903321	----------	----------	----------
163429	----------	-----T----------	----------
B19V GENOTYPE 2	----------	----------	----------
207458	----------	----------	----------
B19V GENOTYPE 3	----------	----------	----------

Fig. 3 Sequence of primers and probe-binding region of the Roche (a) and the in-house (b) B19V DNA assays. The B19V genotype 1 sequence AF162273 was used for reference. B19V sequences of samples 903321, 163429 and 207458 were aligned with the corresponding regions from B19V genotype 2 (A6 strain; AY064476), B19V genotype 3 (V9 strain; AX003421), and the B19V DNA run control. The length of both primers of the Roche test is assumed to be 25 bp. Identical nucleotides as compared to the reference sequence are indicated (-).

failure of the B19V genotype 2 sample 207458. In addition, the sequence of 207458 harbours three mismatches in the forward primer and the already mentioned C→T mismatch in the reverse primer. Of note, Fig. 3a also shows that the six mismatches in the probe-binding region are probably the main reason why the Roche assay is unable to detect B19V genotype 3 strains.

With respect to the in-house assay, sequence analysis of sample 163429 (B19V genotype 1) revealed a mismatch (C→T) in the probe-binding region (Fig. 3b). This mismatch appears to have led to a dramatic destabilization and a decreased hybridization temperature of the TaqMan probe. Indeed, the amplification signal of this sample could be partly restored when the standard annealing temperature of 60 °C

was lowered to 58 or 56 °C (data not shown). The same polymorphism was identified in a commercial factor VIII preparation (coded F8-87-A) produced in the USA with an expiry date of 1987. It was found to give a very shallow amplification plot very similar to that observed for the plasma 163429 (Fig. 1a).

Evaluation of a modified version of the in-house TaqMan assay

The in-house B19V DNA assay was performed using two different versions of the TaqMan probe. The original probe [5] was compared with a modified version containing LNA bases. In the modified probe, LNA bases were incorporated away from the site of the C→T polymorphism observed in strains 163429 and F8-87-A. The LNA bases were included to enhance hybridization to the target sequence by increasing thermal duplex stability and resulting in improvement of the amplification plot. Figure 1a,b shows the results where the two versions of the probe are compared. These amplification plots for strains 163429 and F8-87-A now appear very similar to the wild-type samples. There were no differences observed in the amplification and detection of the B19V controls whether the original version of the TaqMan probe was used or the one containing LNA bases.

Discussion

We applied a commercial (Roche) and an in-house B19V NAT assay for the prescreening of more than 2·6 million donations. The Roche assay was developed prior to the identification of B19V genotypes 2 and 3 and therefore only detects B19V genotype 1. The in-house assay was designed to include genotypes 2 and 3. Three high load B19V DNA samples were identified that gave discordant results between the two B19V DNA assays. Two of these samples (903321 and 163429) were classified as strains of B19V genotype 1. One of them (207458) was classified as B19V genotype 2 strain. To our knowledge, this is the second publication on B19V genotype 2 DNA in a donation of European origin. The first report came from Germany [18]. The conclusion from our study is that new, high viral load B19V genotype 2 infections are rare among blood donors tested by Sanquin, with only a single case identified in 2·6 million donations. This study confirmed the results from an earlier study, in which 321 manufacturing pools (representing more than 950 000 donations from The Netherlands) were tested with a genotype 2- and 3-specific PCR assay and no reactive pools were found (data not shown).

Several previous studies indicated that the prevalence of B19V genotypes 2 and 3 is very low among blood donors from Europe. Heegaard *et al.* [19] found no B19V genotype 3 sequences in 100 000 Danish blood donations. Hokynar *et al.* [14] analysed 140 160 Finnish blood donations and did not reveal any B19V genotype 2 or 3 positive donations. Candotti *et al.* [20] screened donations from the UK and sub-Saharan Africa for the presence of B19V genotypes. Genotype 3 B19V was found to be prevalent in donations from Ghana. Donations originating from the UK, Malawi and South Africa only harboured B19V genotype 1 sequences. Baylis *et al.* [5] tested 52 plasma pools from nine different manufacturers and did not detect any B19V genotype 2 or 3 sequences. These manufacturing pools were sourced from donations collected in Europe and North America. The study of Gierman *et al.* [21] representing a total of 1·5 million donations for US source plasma did also not reveal any B19V genotypes 2 and 3 sequences.

B19V genotype 2 sequences have been sporadically found in final container plasma products. Schneider *et al.* [22] reported B19V genotype 2 sequences in five out of 202 (2·5%) batches of clotting factor concentrates. Recent studies in looking at the persistence of B19V in tissue samples collected in Europe have suggested that in those people born before 1950, either genotype 1 or 2 B19V were found to be present, while those born after this date were predominantly infected with genotype 1 B19V [23]. This may explain why genotype 1 B19V is found so widely in the current blood donor population. This study focused on high load B19V infections rather than low load persistent infections. As the prevalence of low loads of B19V DNA in blood donors is around 1% [20], our study cannot exclude that there might be a significant number of B19V genotype 2 persistent infections, especially in older blood donors.

From the previously published studies [5,12,14], it was already known that the Roche assay was unable to detect B19V genotypes 2 and 3 variants. This study unravels the molecular reasons for this detection failure. With respect to both genotypes 2 and 3 B19V, there are three mismatches in the region of the forward primer. In the case of the reverse primer, there is a single mutation in genotype 3, while there are two mutations for genotype 2; one of these mutations is located at or very near the 3′-end of the primer region. This accounts for observations made in our previous study and in this present one, where there is a reduction in the amplification of genotype 2, when analysed by gel electrophoresis and compared to genotypes 1 and 3 [5]. While all three genotypes are amplified in the PCR, genotypes 2 and 3 are not detected in the real-time assay format, generating no amplification plots. This failure is a consequence of six mismatches found to be present in the region bound by the hybridization probes with these virus genotypes.

Two discrepant samples were classified as B19V genotype 1. Sample 903321 was under-quantified by 2 logs in the Roche assay due to one mismatch at or near the 3′-end of the reverse primer. The other sample 163429 was not detected by the in-house assay because of one mismatch (C→T) in the probe-binding region. This B19V polymorphism was also

detected in a clotting factor VIII concentrate manufactured in the 1980s. Recently, Baylis *et al.* [24] showed the effects of certain mutations in the binding site for TaqMan hydrolysis probes. The conclusion of this study is that the amplification signal correlates with the number of mismatches present in the hydrolysis probe. A single mismatch (G→A) in the wild-type probe-binding region only had a minor effect on the amplification signal. Where four mismatches were present, no amplification signal was observed. Interestingly, none of the described mismatches were C→T changes. This might explain the more dramatic results of our B19V variant where a single mismatch (C→T) caused an unexpectedly dramatic effect. Detection problems due to one C→T mismatch in the TaqMan probe have been described by Teupser *et al.* [25]. The C→T mismatch found in this study led to the misclassification of a polymorphism in the cholesteryl ester transfer protein. It appears that this particular mismatch can lead to dramatic destabilization and decreased hybridization temperature of the TaqMan probe. It is likely that the position of the mismatch within the probe and the adjacent nucleotides also plays a role. The in-house assay was designed to a region within the NS1 gene conserved between all known genotypes of B19V [5]. The identification of a polymorphism within this conserved region was unexpected. In order to improve the robustness of the in-house assay, the TaqMan probe was modified to incorporate LNA bases that counter for the effect of the C→T mutation by increasing the thermal duplex stability. In preliminary studies, the specificity and dynamic range of the test appear not to be impaired by the introduction of these modified nucleotides and more extensive validation studies are in progress.

Our study also shows that amplification curves generated with real-time PCR assays should be interpreted with great care. Sequencing analysis should be performed where unusual amplification patterns are observed.

A systematic approach to find molecular variants of B19V, undetectable or under-quantified with an established PCR assay can be achieved by using a second independent PCR assay. This study of B19V variants uses a generic extraction of nucleic acid. Subsequently, two different parts of the NS1 region are amplified to detect and quantify B19V.

It has recently been shown that the variation within the B19V genome is greater than that was previously believed [26]. Indeed, it was found that B19V had a surprisingly high rate of evolutionary change, at approximately 10^{-4} nucleotide substitutions per site per year. These observations, together with the data presented in this study, indicate that the variation in the B19V genome should be carefully monitored. Constant monitoring of B19V sequences in the population will help to ensure that primers and probes, based upon conserved sequences, are still applicable when variant viruses are identified. The nature of the genetic variation ranges from the identification of new genotypes, through to single nucleotide polymorphisms that can affect assay performance. Where new viral variants are identified, and this extends beyond B19V, kit manufacturers are faced with validation and regulatory challenges to vary existing tests or introduce new ones. Such changes impact upon the end-users implementing the tests. Prevalence studies of virus variants may be useful to determine whether it is necessary to broaden the scope of a particular test.

In summary, we identified one B19V genotype 2 strain and two B19V genotype 1 strains that were under-quantified or not detected at all by a commercial and an in-house B19V DNA assay while screening more than 2·6 million blood donations in plasma pools. As compared to B19V genotype 2 strains, the prevalence of B19V genotype 1 variants not detected by commercial or in-house assays might be in the same range or even higher.

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別紙様式第 2-1

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別紙 3-2

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2007 年 10 月 11 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Improved detection of acute parvovirus B19 infection by immunoglobulin M EIA combined with a novel antigen EIA. Corcoran, A. et al, Vox Sanguinis, 93, 216-222 (2007).	公表国 アイルランド	
販売名（企業名）						

研究報告の概要	使用上の注意記載状況・その他参考事項等
本稿では，ヒト血漿中における B19 ウイルス抗原を直接検出する酵素免疫測定法（EIA）について報告した。アッセイの検出限界は 10 pg/mL の組換え VP2 カプシドタンパク質であり，理論的には 1ml 中に 1.9x10E6 個の B19V 粒子を検出できることに相当する。当該アッセイを用いて 2003 年 2 月から 2004 年 7 月の間にオランダにおいて無症候ドナーから採取した 70 のウイルス血症性の献血検体(B19 DNA の濃度が 10E6 IU/mL を超える）を検査した。これらの検体は，低 pH の状態では B19 検出が大幅に増加することがわかった。興味深いことに，B19 抗原の検出は B19 の抗体（IgM 又は IgG）が共存することによって左右されなかった。さらに，本アッセイではヒトパルボウイルスの遺伝子型 1，2 及び 3 を同等に検出した。また，B19 抗原の EIA 法及び B19 IgM の EIA 法を合わせることで，B19V 感染初期と思われる（IgM が検出される）検体の 91%を検出した。B19 IgM 検出と B19 抗原検出を組み合わせた EIA 法は PCR に替わる最近の B19 感染の有効な検出法となると思われる。	BYL-2008-0298

報告企業の意見	今後の対応
本論文では，ヒト血漿中における B19 ウイルス抗原を直接検出する酵素免疫測定法（EIA）について報告した。抗原には P2 カプシドタンパク質を用いた。特に，B19 IgM 検出を組み合わせることで，効果的に感染初期のサンプルを検出可能であることを示している。本方法は，B19 ウイルス 1，2 及び 3 型を検出可能であり，測定感度も十分に高く，PCR に変わる測定方法として期待される。 弊社のポリグロビン N の製造に使用されるミニプール血漿においては，ヒトパルボウイルス B19 に対する NAT を実施しており，10E5 IU/mL 以上が確認された場合は，そのミニプール血漿は製造工程から除去している。現在の科学水準では，ヒトパルボウイルス B19 を確実に不活化する方法は存在しないため，感染リスクを完全に排除することはできないが，伝播の可能性は非常に低いと考える。	本稿で報告されたような，大規模試験に利用可能な測定法に関して今後とも情報収集に努める。

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ORIGINAL PAPER

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

A. Corcoran,[1] S. Kerr,[1] G. Elliott,[1] M. Koppelman[2] & S. Doyle[3]

[1]Biotrin International, Dublin, Ireland
[2]Sanquin Diagnostic Services, Plesmanlaan 125, Amsterdam, The Netherlands
[3]National Institute for Cellular Biotechnology, National University of Ireland Maynooth, Co. Kildare, Ireland

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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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

Correspondence: Amanda Corcoran, Biotrin International, 93 The Rise, Mount Merrion, Co. Dublin, Ireland
E-mail: amanda.corcoran@biotrin.ie

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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