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Parvovirus B19 genotypes 1 and 2 detection with real-time polymerase chain reaction assays

M. H. G. M. Koppelman,[1] I. G. H. Rood,[1] J. F. Fryer,[2] S. A. Baylis[2] & H. T. M. Cuypers[1]

[1]Sanquin, Diagnostic Services Division, Amsterdam, The Netherlands
[2]Division of Virology, National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK

Vox Sanguinis

Background and Objectives Parvovirus B19 (B19V) DNA screening has been introduced to comply with European regulations for certain plasma products. Current commercial and some in-house B19V DNA assays fail to detect or under-quantify the recently identified genotypes 2 and 3. In this report, we describe 2-year experience with B19V DNA screening using the commercial assay from Roche (detecting only genotype 1) combined with an in-house assay (detecting genotypes 1, 2 and 3). This dual testing approach enables the identification of molecular variants of B19V.

Materials and Methods Between 2005 and 2007, approximately 2·6 million plasma donations were screened for B19V DNA loads exceeding 10^6 IU/ml using the Roche and the in-house real-time polymerase chain reaction assay.

Results A total of 232 plasma units were identified with B19V DNA loads above 10^6 IU/ml. Concordant results were observed for the majority of B19V positive samples; however, three of these showed discrepant results between the two assay systems. One was a B19V genotype 2 strain not detected by the Roche assay; another was a B19V genotype 1 strain with a mismatch in the 3′-end of the reverse primer and therefore under-quantified by the Roche assay; and the third one was also a B19V genotype 1 strain that gave an unusual amplification plot in the in-house assay due to a mismatch in the probe-binding site.

Conclusions New, high viral load, B19V genotypes 2 and 3 infections are rare in blood donors tested by Sanquin. One case was found while testing 2·6 million donations. The prevalence of B19V genotype 1 variants not detected by commercial or in-house assays might be in the same range or even higher than the prevalence of B19V genotype 2 viruses, which remain undetected.

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Introduction

In 2004, European regulations came into force to limit the potential parvovirus B19 (B19V) burden in plasma pools for the manufacture of human anti-D immunoglobulin and pooled human plasma treated for virus inactivation [1]. The level of B19V DNA in these manufacturing pools should not exceed a threshold concentration of 10 000 IU/ml. To comply with these requirements, the plasma fractionation industry set up a screening system to prevent plasma units with high B19V DNA loads from entering large manufacturing pools. Most of the industry have introduced systems in which donations are prescreened in test pools of 480–960 donations [2–4]. When the B19V DNA level in a test pool exceeds the defined exclusion limit, the index donation is traced using a break-down protocol to smaller test pools.

Correspondence: M. H. G. M. Koppelman, Sanquin Diagnostic Services Division, Plesmanlaan 125, 1066 CX, Amsterdam, The Netherlands
E-mail: m.koppelman@sanquin.nl

Donor screening for B19V DNA requires quantitative nucleic acid amplification tests (qNAT) because donations exceeding a defined exclusion limit must be identified and subsequently removed. Several in-house and commercially available qNAT assays have been developed for this purpose between 1999 and 2004 [2,5,6]. Two commercial assays from Artus (RealArt Parvovirus B19 LightCycler PCR kit) and Roche (LightCycler Parvovirus B19 DNA quantification kit) are currently on the market for quantitative detection of B19V DNA.

In parallel with the development of qNAT assays for B19V DNA, several virus strains have been identified that show greater sequence diversity than that was previously recognized for B19V [7–10]. Phylogenetic analysis of B19V and these related variants showed that the viruses fall into three genotypes [10]. The prototypical sequences for B19V fall into genotype 1; genotype 2 viruses include A6 [7] and LaLi [9], while genotype 3 viruses include V9 [8] and D91-1 [10]. In the eighth report from the International Committee on the Taxonomy of Viruses (ICTV), A6, LaLi and V9 have all been classified as strains of B19V [11]. The consequence of this official classification is that detection of these two new genotypes of B19V is now mandatory according to the European regulations for 'in process testing' of manufacturing pools for B19V DNA.

Detection of these recently classified B19V genotypes 2 and 3 with commercial assays and in-house assays is limited. Several publications and the proficiency testing studies (PTSs) organized by European Directorate for the Quality of Medicines (EDQM) showed that commercial B19V DNA assays and several in-house assays have issues with the detection and/or quantification of B19V genotypes 2 and 3 strains. The Artus B19V DNA assay reliably quantified B19V genotypes 1 and 2 and some genotype 3 subtypes. However, one of the genotype 3 B19V subtypes is under-quantified by at least 3 logs [5,12,13]. The Roche assay reliably quantifies B19V genotype 1, but fails to detect genotypes 2 and 3 [2,5,12,14]. These findings are also reflected in the recent PTSs [12]. In the study performed in 2004 (PTS052), 56% of the laboratories that participated missed the B19V genotype 2 sample. The study organized in 2005 (PTS064) showed that 41% of the participants missed the B19V genotype 2 sample. In the latter study, 25% of laboratories using in-house assays were unable to detect the B19V genotype 2.

The Roche B19V DNA assay has been used for screening all plasma, in test pools of 480 donations [2]. As the Roche assay fails to detect genotypes 2 and 3 of B19V, an additional assay able to detect and quantify all three genotypes of B19V was introduced in 2005 [5]. Currently, all donations are tested in parallel with these two B19V DNA assays. This study reports the results of 2-year experience using the dual testing approach on more than 2·6 million donations.

Materials and methods

B19V DNA testing of plasma

Between March 2005 and March 2007, Sanquin tested approximately 2·6 million blood donations for B19V DNA load. Plasma was tested in test pools of 480 donations and in manufacturing pools. Test pools with B19V DNA loads above 5000 IU/ml were subjected to further testing to track down the index donation(s).

Commercial and in-house B19V DNA real-time polymerase chain reaction amplification

Nucleic acid from manufacturing pools, test pools and individual donations (0·1–1·0 ml plasma input) was isolated using the NucliSens extractor (NucliSens, bioMerieux, Boxtel, The Netherlands) [15].

Two real-time polymerase chain reaction (PCR) assays were performed with the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) on the same nucleic acid extract. The first assay was the B19V DNA quantification assay from Roche. This assay amplifies a fragment within the non-structural protein (NS1) gene [2]. The amplicons are detected with hybridization probes. The second assay was an in-house developed B19V DNA assay with primers (EVF and EVR; see Table 1) and TaqMan probes in the NS1 region. This assay was adapted from Baylis et al. [5] and reliably detects and quantifies B19V genotypes 1, 2 and 3. To improve the robustness of the in-house TaqMan assay, a modified hydrolysis probe was included. The modified probe had an identical DNA sequence; however, locked nucleic acid (LNA) bases were incorporated at specific sites [16,17]. The sequence of the modified TaqMan probe is as follows, with LNA bases shown underlined: 5′ (FAM)-AAC.CCC.GCG.CTC.TAG.TAC-(BBQ3) 3′. The sensitivity (95% detection limit) was similar for both B19V DNA assays and was approximately 100 IU/ml (data not shown).

B19V DNA sequence analysis

Purified PCR products were sequenced with the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (version 3·0) according to the manufacturer's instructions (Applied Biosystems/Hitachi, Nieuwerkerk a/d IJssel, The Netherlands). Sequence analysis was performed on the ABI PRISM 3130xl Genetic Analyser. Table 1 shows the panel of primers used for sequencing in this study.

Determination of target and probe-binding regions in the Roche B19V DNA assay

In order to determine the region in the B19V that is targeted by the Roche assay, the assay was performed using the 10 000 IU/ml

Table 1 Panel of primers for sequencing of B19V (NS1–VPu region)

Primer (forward or reverse)	Sequence (5′→3′)	Nucleotide position in AF162273	Reference
P2 (f)	AAA.CTA.GCA.ATT.TAT.AAA.GC	1392–1411	This study
P3 (f)	TGG.ATT.GAT.AAA.AAA.TGT.GG	1551–1570	This study
P4 (f)	TTG.GTG.GTC.TGG.GAT.GAA.GG	1716–1735	This study
PVB-3 (f)	ATA.AAC.TAC.ACT.TTT.GAT.TTC.CCT.G	2052–2076	[20]
EVF (f)	AAT.GCA.GAT.GCC.CTC.CAC	2082–2099	[5]
EVR (r)	ATG.ATT.CTC.CTG.AAC.TGG.TCC	2254–2274	[5]
PV-1 (f)	GGA.CCA.GTT.CAG.GAG.AAT.CAT	2254–2274	This study
PV-2 (f)	GCT.TGG.TAT.AAT.GGA.TGG.AA	2481–2500	This study
PV-3 (r)	CCA.GAC.AGG.TAA.GCA.CAT.TT	2583–2602	This study
PV-4 (f)	TTT.GAC.TTA.GTT.GCT.CG	2800–2816	This study
PV-5 (r)	TGA.AAA.TGA.TGA.CTA.TAT.A	2849–2867	This study
B19SR (r)	CCA.GGC.TTG.TGT.AAG.TCT.TC	2959–2978	[20]

B19V run control. In this instance, the internal control from the kit was not added prior to extraction. DNA sequence analysis was performed to identify the location of the B19V amplicon in the viral genome. The sequence of the probe-binding region for the B19V was determined by comparing the sequence of the B19V amplicon with the sequence of the internal control amplicon (amplified in the absence of B19V DNA).

B19V genotyping

Viral DNA for genotyping was obtained by PCR amplification of a 1587-bp fragment spanning the NS1–VP1u junction in the B19V genome with primers P2f and B19SR (see Table 1). This fragment overlaps the PCR fragments amplified by the Roche and the in-house assay. Both DNA strands were sequenced with the panel of 12 sense and antisense oligonucleotides.

Phylogenetic analysis was performed using the Vector NTI 10·1·1 software package (Invitrogen, Carlsbad, CA, USA) and the Molecular Evolutionary Genetics Analysis software (MEGA2·1: Arizona State University, Tempe, AZ, USA). Neighbour-joining phylogenetic analysis was performed on a 1536-bp fragment (nucleotides 1436–2971 in AF162273). Nucleotide distances were calculated using the Kimura 2-parameter model using the bootstrap test with 1000 replicates.

The following B19V sequences from GenBank were used as reference sequences: *B19V genotype 1*, AF161226, AF162273, AY504945, DQ293995, M24682, M13178; *B19V genotype 2*, AJ717293, AY064476, AY064475, AY044266, AY903437, DQ333426, EF216869; *B19V genotype 3*, AJ249437, AY582125, AY647977, AY083234, AX003421, DQ234769, DQ234779, DQ408305, NC-004295.

Nucleotide sequence accession numbers

The GenBank accession numbers of the nucleotide sequences of the B19V variants analysed in this study are EF151136 (strain 163429), EF151137 (strain 903321), EF151138 (strain 207458) and EF216868 (strain F8-87-A).

Results

Identification of B19V genotypes 1 and 2 strains in plasma samples

Between March 2005 and March 2007, 2·6 million donations were prescreened for B19V DNA using dual assay testing approach. B19V DNA screening of test pools aims to identify donations with B19V DNA loads above 10^6 IU/ml. During the 2-year study period, 232 donations were identified with B19V DNA levels exceeding 10^6 IU/ml. Thus, donations with loads above the exclusion level occur with a frequency of about 1 in 11 000 in this donor population. In three cases (1·3%), discrepant results between the Roche and the in-house B19V DNA test were found (Table 2). Plasma sample 207458 is a donation undetectable by the Roche assay; however, it is found to have a viral load of 3×10^7 IU/ml in the in-house assay. The second sample, 163429, is a donation containing 10^8 IU/ml B19V DNA in the Roche assay. In contrast, this donation was hardly detectable by the in-house assay in the original test pool of 480 donations. Also at the individual

Table 2 Discrepant cases between the Roche and the in-house parvovirus B19 (B19V) DNA assay

Sample	Viral load (Roche assay)	Viral load (in-house assay)
207458	Not detectable	3×10^7 IU/ml
163429	1×10^8 IU/ml	Hardly detectable and not quantifiable[a]
903321	7×10^2 IU/ml	3×10^4 IU/ml

[a]Individual donation testing revealed a shallow amplification curve (see Fig. 1a).

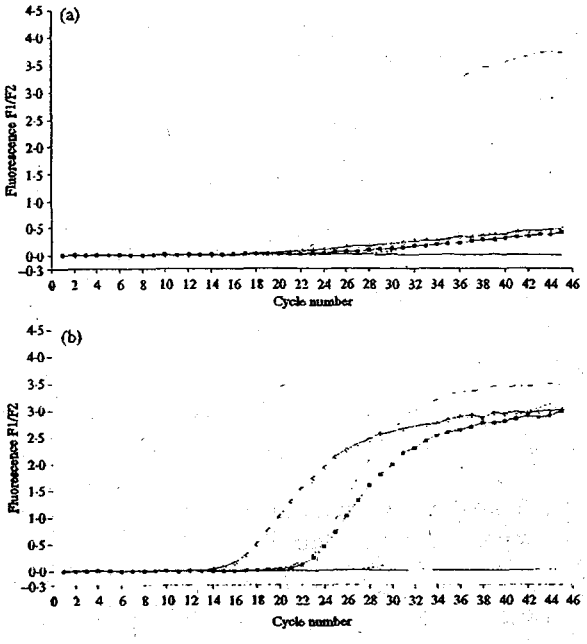

Fig. 1 Amplification plots obtained with the in-house assay under normal conditions using a TaqMan probe labelled with FAM/TAMRA and used at 0·2 μM [5] (a) and the assay performed under normal conditions using a locked nucleic acid (LNA) probe labelled with FAM/BBQ3 and used at 0·02 μM (b).

donation level, it was hardly detectable when the routine procedure (fit-points method) was used for calculation of the crossing-point. When the crossing-point was calculated with an alternative method (second derivative maximum method), the value of the crossing-point was comparable to the one obtained with the Roche assay. The amplification plot of sample 163429 obtained with the in-house assay showed a very shallow amplification signal (Fig. 1a). The third case, 903321, is a plasma sample with an almost 100-fold difference in load between the Roche and the in-house assays.

B19V genotyping

In each case, the sequence of a 1536-bp fragment overlapping the NS1–VP1 region of B19V was determined and aligned with B19V genotype 1, 2 and 3 sequences from GenBank. This alignment was used to construct a phylogenetic tree as shown in Fig. 2. The tree clearly shows that cases 163429 (not detected by the in-house assay) and 903321 (not detected by the Roche assay) are strains of B19V genotype 1 and that case 207458 (not detected by the Roche assay) is a B19V genotype 2 strain.

Molecular basis of the discrepant results

Initially, it was necessary to determine the region of the B19V genome targeted by the Roche assay. The amplified B19V product from the Roche assay was sequenced and found to correspond to a 177-bp region of the B19V NS1 gene (nucleotides 1552–1708 of the reference strain HV; accession number AF162273). It was assumed that the primers used in the Roche assay were 25 bp in length. In order to identify the probe-binding region for the Roche assay, the DNA sequence of the internal control amplicon from the Roche assay was determined. This revealed that the internal control corresponded to the wild-type B19V sequence with the exception of a 52-bp insert derived from the human telomerase RNA gene (nucleotides 881–932; accession number AF047386). This insert is four nucleotides longer than the corresponding wild-type B19V sequence it has replaced (i.e. nucleotides 48–94 of the wild-type B19V PCR fragment). This 46-bp sequence is considered to represent the hybridization probe-binding region of the Roche B19V DNA assay.

Figure 3 shows sequence alignments of the relevant regions of the PCR fragments (primer and probe-binding sites) of the Roche assay (a) and the in-house assay (b).

With respect to the Roche assay, there are a considerable number of mismatches in both the primer and the probe-binding regions of B19V genotypes 2 and 3 sequences (Fig. 3a). The forward primer contains three mismatches. The reverse primer contains one mismatch in the B19V genotype 3 strain and two mismatches in the B19V genotype 2 strain. Notably, one mismatch (C→T) in the B19V genotype 2 reverse primer sequence is located at or near the 3′-end. Mutations at the 3′-end of a primer may result in no amplification. This is the most likely reason why sample 903321 is not detected by the Roche assay. Although sample 903321 is a B19V genotype 1 strain, it harbours the B19V genotype 2 typical C→T mismatch in the reverse primer. In this case, the mismatch probably leads to inefficient amplification rather than no amplification at all. This has been reported for the A6 genotype 2 B19V strain [5]. Inefficient amplification could explain the 100-fold difference between the Roche and the in-house assay. In order to investigate this further, sample 903321 containing 30 000 IU/ml (in-house assay) and the run control containing 10 000 IU/ml were amplified, using the Roche assay in the absence of the internal control. Both amplicons were analysed by agarose gel electrophoresis and staining with SYBR green. While it was clear that amplification had occurred, the stained band of sample 903321 was of reduced intensity compared to the run control, suggesting that the C→T mutation caused inefficient amplification (data not shown).

The probe-binding region of the Roche assay contains six mismatches in the B19V genotypes 2 and 3 sequences (Fig. 3a). These six mismatches only partly explain the detection

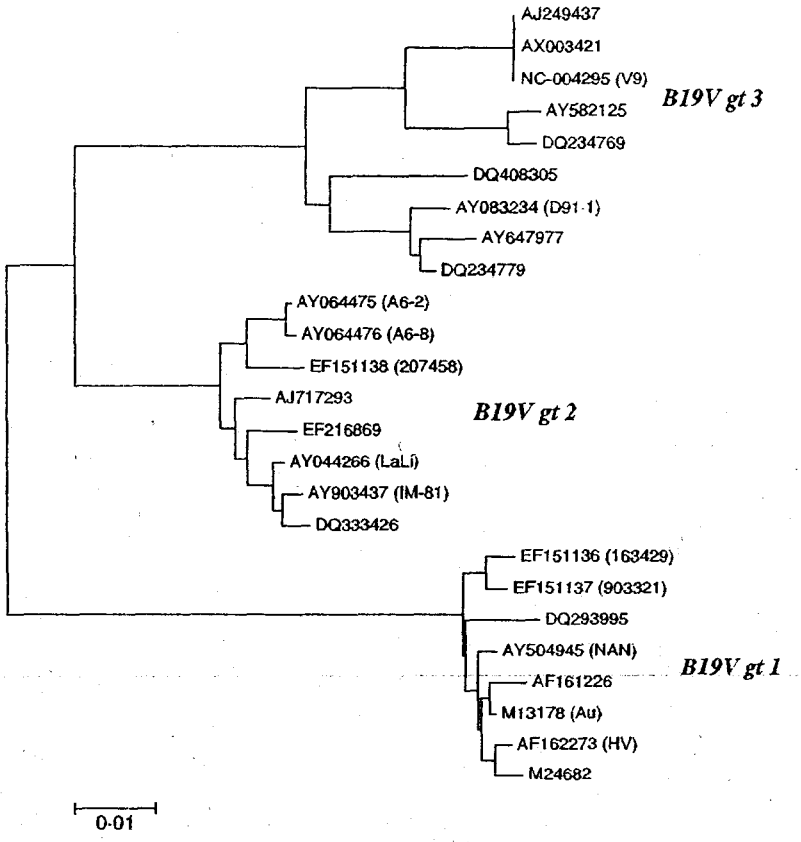

Fig. 2 Phylogenetic tree of parvovirus B19 (B19V) DNA sequences from three B19V variants (903321, 163429 and 207458) and B19V DNA sequences with published genotypes from GenBank. The sequence of a 1536-bp fragment corresponding to part of the NS1–VP1 region was used to create the tree.

(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

医薬品
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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。

26