did, however, not reveal any significant difference in B19V IgG titers. This suggests that lowering the B19V PCR test limit to 10^4 IU per mL would not impair the B19V neutralization capacity contained in plasma pools and thus also IVIG products. Also, although our data show that even at the lowest B19V IgG concentrations determined for a plasma pool of greater than 10^4 IU per mL B19V are neutralized, the combination between uncompromised B19V antibody levels and further reduced B19V loads in plasma manufacturing pools might even enhance the safety margins of plasma products as primarily afforded by the virus reduction capacity of their manufacturing processes, particularly for IVIG that has already enjoyed a longstanding history of safety with respect to B19V transmission[33] and other antibody-containing products.

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REFERENCES

1. Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. Virus taxonomy: classification and nomenclature of viruses—eighth report of the International Committee on the Taxonomy of Viruses. San Diego: Elsevier Academic Press; 2005.
2. Liefeldt L, Plentz A, Klempa B, Kershaw O, Endres AS, Raab U, Neumayer HH, Meisel H, Modrow S. Recurrent high level parvovirus B19/genotype 2 viremia in a renal transplant recipient analyzed by real-time PCR for simultaneous detection of genotypes 1-3. J Med Virol 2005;75:161-9.
3. Schneider B, Becker M, Brackmann HH, Eis-Hubinger AM. Contamination of coagulation factor concentrates with human parvovirus B19 genotype 1 and 2. Thromb Haemost 2004;92:838-45.
4. Candotti D, Etiz N, Parsyan A, Allain JP. Identification and characterization of persistent human erythrovirus infection in blood donor samples. J Virol 2004;78:12169-78.
5. Cossart YE, Field AM, Cant B, Widdows D. Parvovirus-like particles in human sera. Lancet 1975;1(7898):72-3.
6. Young NS, Brown KE. Parvovirus B19. N Engl J Med 2004;350:586-97.
7. Pankuweit S, Ruppert V, Eckhardt H, Strache D, Maisch B. Pathophysiology and aetiological diagnosis of inflammatory myocardial diseases with a special focus on parvovirus B19. J Vet Med B Infect Dis Vet Public Health 2005;52:344-7.
8. Eid AJ, Brown RA, Patel R, Razonable RR. Parvovirus B19 infection after transplantation: a review of 98 cases. Clin Infect Dis 2006;43:40-8.
9. Red book: report of the Committee on Infectious Diseases. 27th ed. Elk Grove Village (IL): American Academy of Pediatrics; 2006.
10. Heegaard ED, Brown KE. Human parvovirus B19. Clin Microbiol Rev 2002;15:485-505.
11. Solheim BG, Rollag H, Svennevig JL, Arafa O, Fosse E, Bergerud U. Viral safety of solvent/detergent-treated plasma. Transfusion 2000;40:84-90.
12. Bonvicini F, Gallinella G, Cricca M, Ambretti S, Delbarba S, Musiani M, Zerbini M. Molecular testing for detection of in vitro infectivity of plasma pools contaminated with B19 virus. J Med Virol 2004;74:272-6.
13. Daly P, Corcoran A, Mahon BP, Doyle S. High-sensitivity PCR detection of parvovirus B19 in plasma. J Clin Microbiol 2002;40:1958-62.
14. Schmidt I, Blumel J, Seitz H, Willkommen H, Lower J. Parvovirus B19 DNA in plasma pools and plasma derivatives. Vox Sang 2001;81:228-35.
15. Wong S, Brown KE. Development of an improved method of detection of infectious parvovirus B19. J Clin Virol 2006;35:407-13.
16. Prikhod'ko GG, Vasilyeva I, Reyes H, Wong S, Brown KE, Jameson T, Busby TF. Evaluation of a new LightCycler reverse transcription-polymerase chain reaction infectivity assay for detection of human parvovirus B19 in dry-heat inactivation studies. Transfusion 2005;45:1011-9.
17. Caillet-Fauquet PG, Draps ML, de Hougardy VL, Laub R. An assay for parvovirus B19 neutralizing antibodies based on human hepatocarcinoma cell lines. Transfusion 2004;44:1340-3.
18. Blumel J, Schmidt I, Willkommen H, Lower J. Inactivation of parvovirus B19 during pasteurization of human serum albumin. Transfusion 2002;42:1011-8.
19. Yunoki M, Tsujikawa M, Urayama T, Sasaki Y, Morita M, Tanaka H, Hattori S, Takechi K, Ikuta K. Heat sensitivity of human parvovirus B19. Vox Sang 2003;84:164-9.
20. Blumel J, Eis-Hubinger AM, Stuhler A, Bonsch C, Gessner M, Lower J. Characterization of Parvovirus B19 genotype 2 in KU812Ep6 cells. J Virol 2005;79:14197-206.
21. Shimomura S, Komatsu N, Frickhofen N, Anderson S, Kajigaya S, Young NS. First continuous propagation of B19 parvovirus in a cell line. Blood 1992;79:18-24.
22. Nguyen QT, Wong S, Heegaard ED, Brown KE. Identification and characterization of a second novel human erythrovirus variant, A6. Virology 2002;301:374-80.
23. Kerr JR, O'Neill HJ, Deleys R, Wright C, Coyle PV. Design and production of a target-specific monoclonal antibody

to parvovirus B19 capsid proteins. J Immunol Methods 1995;180:101-6.
24. Ozawa K, Ayub J, Hao YS, Kurtzman G, Shimada T, Young N. Novel transcription map for the B19 (human) pathogenic parvovirus. J Virol 1987;61:2395-406.
25. PPTA. PPTA voluntary standard parvovirus B19. Report 017677. Annapolis (MD): Plasma Protein Therapeutics Association; 2001 Feb 8. p. 1-2.
26. European Pharmacopoeia Expert Group. Human anti-D immunoglobulin. Pharmeurope 2002;14:81.
27. Nguyen QT, Sifer C, Schneider V, Allaume X, Servant A, Bernaudin F, Auguste V, Garbarg-Chenon A. Novel human erythrovirus associated with transient aplastic anemia. J Clin Microbiol 1999;37:2483-7.
28. Miyagawa E, Yoshida T, Takahashi H, Yamaguchi K, Nagano T, Kiriyama Y, Okochi K, Sato H. Infection of the erythroid cell line, KU812Ep6 with human parvovirus B19 and its application to titration of B19 infectivity. J Virol Methods 1999;83:45-54.
29. Yunoki M, Urayama T, Tsujikawa M, Sasaki Y, Abe S, Takechi K, Ikuta K. Inactivation of parvovirus B19 by liquid heating incorporated in the manufacturing process of human intravenous immunoglobulin preparations. Br J Haematol 2005;128:401-4.
30. Davenport RJ, Geohas G, Cohen S, Beach K, Lazo A, Lucchesi K, Pehta J. Phase IV Study of Plas+SD: hepatitis A (HAV) and parvovirus B19 (B19) safety results. Blood 2000;96 Suppl 1:451a.
31. Brown KE, Young NS, Alving BM, Barbosa LH. Parvovirus B19: implications for transfusion medicine. Summary of a workshop. Transfusion 2001;41:130-5.
32. Feldschuh J, Enson Y. Prediction of the normal blood volume relation of blood volume to body habitus. Circulation 1977;56:605-12.
33. Farrugia A. Risk of parvovirus infection by immunoglobulin. Br J Haematol 2003;121:955-6.

別紙様式第 2-1

別紙 3-1

医薬品
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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	Parvovirus B19 genotypes 1 and 2 detection with real-time polymerase chain reaction assays. Koppelman, M.H.G.M. et al, Vox Sanguinis, 93, 208-215 (2007).	公表国 オランダ	
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
2005 年 3 月から 2007 年 3 月の間にオランダで実施された，260 万の血漿成分献血検体を対象とした B19 ウイルス（B19V）のスクリーニング試験の結果を報告した。献血検体は，B19 の遺伝子 1 型のみを検出する Roche 社製の市販の DNA 定量アッセイと，3 種すべての遺伝子型を検出できる社内アッセイ（Sanquin，オランダ）の 2 種類の PCR アッセイで測定した。本試験では 10^6 IU/mL を超えるウイルス価を示す検体を B19V 陽性と判断した。両アッセイの検出限界は 100 IU/mL 前後と同等であった。480 検体からなるテスト用プール，及び製造用プールを測定し，5000 IU/ml 超を示したプールに関して，さらに詳細に測定した。その結果，232 検体（11000 検体につき 1 検体）で B19V が確認（10^6 IU/mL 以上）された。これら B19 陽性検体の大多数ではアッセイ間で一致が見られたが，3 検体（1.3%）では不一致が認められた。ジェノタイピング及び各アッセイで用いたプライマー及びプローブの結合領域の配列解析により，2 検体は B19 遺伝子 1 型に分類され，残りの 1 検体は遺伝子 2 型に分類されることを明らかとした。従って，遺伝子 2 型及び 3 型の保有率はヨーロッパ人ドナーにおいては極めて低いと考えられる。今回の試験は別のグループによる過去の知見を裏付けている。	BYL-2008-0297

報告企業の意見	今後の対応
オランダにおける献血検体中 B19 ウイルスのスクリーニング結果が報告された。異なる PCR アッセイを組み合わせることで，3 種類の B19 ウイルスアイソフォームの検出を可能としている点は，新規性が高く，今後の応用が期待される。大規模スクリーニングの結果，ヨーロッパにおける献血では，11000 検体に 1 検体の割合で B19 ウイルスが検出され，1 検体を除き遺伝子 1 型に分類された。ヨーロッパ人ドナーにおいて 2 型及び 3 型の保有率は極めて低いと考えられた。 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。	現時点で新たな安全対策上の措置を講じる必要はないと考える。

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

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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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Key words: B19V genotype 2, parvovirus B19 DNA, screening, real-time PCR.

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].

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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.

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

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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).

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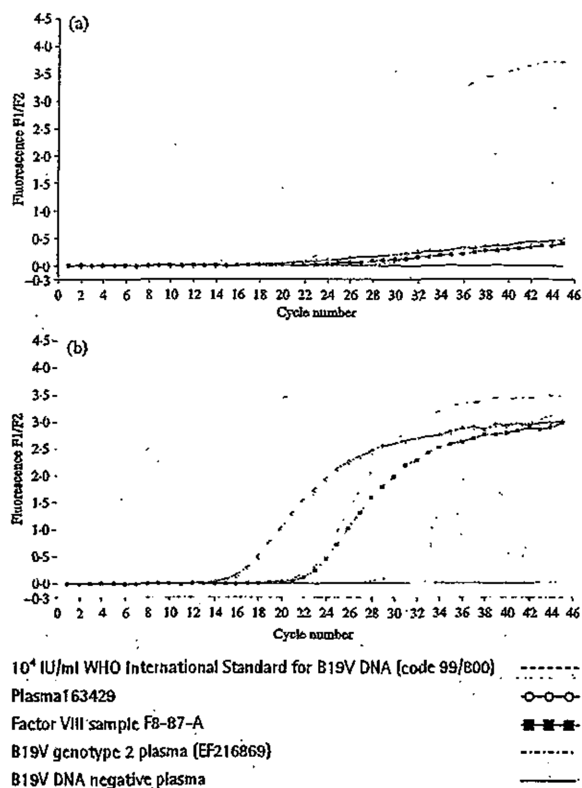

Fig. 1 Amplification plots obtained with the in-house assay under normal conditions using a TaqMan probe labelled with FAM/TAMRA and used at 0·2 μM [5] (a) and the assay performed under normal conditions using a locked nucleic acid (LNA) probe labelled with FAM/BBQ3 and used at 0·02 μM (b).

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

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