

implementation of these regulations and to discuss how best to respond to changes in the molecular epidemiology of viruses.

Classification of B19V

Dr K. Brown [Health Protection Agency (HPA), UK] described the criteria used by the ICTV to classify viruses. Classification by the ICTV does not extend beyond species and no consideration is given to either genotypes or clades. In the case of B19V, it is classified as a member of the *Parvoviridae* family, belonging to the erythrovirus genus. While sequence comparisons are becoming increasingly important in classification, other criteria are considered including mode of replication, virus structure, genomic organization, transcriptional and biological properties. The ICTV has classified the recently identified variant viruses, specifically V9, originally identified in France [8], A6 [9] and LaLi [10] as strains of B19V [6]. The genetic diversity of the B19V strains falls into three well-recognized genotypes [5], which can be confirmed by pairwise sequence identity profiles. Nucleotide divergence is approximately 10–15% between the different genotypes. These B19V strains are clearly distinct from other erythroviruses, such as the primate parvoviruses viruses [pig-tailed macaque parvovirus (PmPV), rhesus macaque parvovirus (RmPV) and simian parvovirus (SPV)], and more distant, tentative members such as bovine parvovirus 3 and chipmunk parvovirus. Dr Brown mentioned that the two recently identified human parvoviruses, that is, human parvovirus PARV4 [11,12] and human bocavirus [13], are quite distinct from B19V and would not be discussed further during the meeting.

Regulatory issues

Dr J.-M. Spieser (EDQM, Strasbourg, France) summarized the B19V test kit meeting held at EDQM on 9 November 2006, in response to differences in the ability of laboratories to detect genotype 2 B19V. Genotype 2 B19V has been identified in plasma pools that are undergoing batch release in Europe. The assay available from Roche is suitable only for the detection of genotype 1 B19V. The Artus (Qiagen, Hamburg, Germany) assay detects genotype 2 and some of the genotype 3 viruses. Both companies are addressing the shortfalls in the current assay kits. Currently, the Official Control Authority Batch Release (OCABR) guidelines require the detection of genotype 1 B19V, and recommend the detection of viruses such as A6 and V9 [14]. The batch release advisory group have endorsed the proposal that the guideline for B19V should be updated to reflect the requirement for the detection of different virus genotypes and be mandated in the Ph. Eur.

Dr M. W. Yu (Center for Biologics Evaluation and Research; CBER, Bethesda, MD, USA) reviewed the US Food and Drug Administration's (FDA) previous discussions on NAT testing for B19V in the USA. Most source plasma fractionators

perform in-process B19V NAT testing, excluding high-titre donations following mini-pool testing. Blood collection establishments voluntarily retrieve and discard in-date components from donors with high titres of B19V DNA, to prevent their use in transfusion recipients. An infusion of a coagulation factor VIII product devoid of any anti-B19V, which was derived from plasma unscreened for B19V by NAT in a mini-pool format, with an overall load of B19V DNA as low as 2×10^4 IU, has been shown to transmit in a seronegative recipient [15]. For manufacturing pool B19V NAT testing, the FDA is currently proposing a limit of $\leq 10^4$ IU/ml for all plasma-derived products. The FDA has reviewed and approved some in-house B19V NAT procedures, for mini-pools and manufacturing pools under the Biologics Licensing Applications or their supplements for plasma derivatives. B19V NAT assays are required to be validated as analytical procedures and should be capable of detecting all virus genotypes. In the future, the FDA may consider B19V testing as donor screening, because of known risks in individuals with chronic anaemia, those who are pregnant or immunocompromised. Such screening would be dependent upon the availability of suitable commercial kits and sufficient resolution time.

Prevalence and clinical properties of different genotypes of B19V

Ms K. Hokynar (Haartman Institute, Helsinki, Finland) described studies where B19V DNA was identified in skin biopsies. Sequence analysis identified more divergent viruses, now recognized as genotype 2 B19V [10]. Analysis of tissue samples from North West Europe failed to identify genotype 3 B19V; however, genotypes 1 and 2 were both readily identified individuals born prior to 1950, while those born after this date were predominantly infected with genotype 1 [16]. *In vitro* studies of the three genotypes showed no differences in infectivity or in the activity of the p6 promoter, which is most efficient in cells permissive for B19V infection, and enhanced by the expression of NS1 [17]. Serological cross-reactivity is observed between B19V genotypes 1 and 2 using recombinant antigens and sera from individuals infected with specific genotypes [17]. All three genotypes of B19V are extremely similar, constituting a single serotype, with amino acid divergence for VP1 no greater than 4%.

Professor S. Modrow (University of Regensburg, Germany) described a clinical case, where a renal transplant recipient developed transient anaemia and arthritis and was diagnosed with B19V. Detailed molecular analysis revealed that the patient was infected with a genotype 2 B19V. A review of the original assays performed showed that there was differential sensitivity for the different genotypes of B19V. The patient showed persistent, high levels of B19V DNA ($> 10^{11}$ genome equivalents (geq)/ml) and episodes of severe anaemia [18]. Treatment with intravenous immunoglobulin (IVIG) lowered

viral loads and resolved anaemia. After 4 years, B19V DNA and anti-B19V IgM antibodies were still detectable. The patient subsequently started to develop anti-B19V IgG antibodies. Both IgG reactivity and avidity were comparable in sera from genotypes 1 and 2 B19V-infected individuals, when challenged by enzyme-linked immunosorbent assay (ELISA) using antigen from the VP1-unique region from all three genotypes. It was noted that this case presented in a very similar way to ones seen with a genotype 1 B19V infection.

Epidemiological studies of B19V infection in blood donors, pregnant women and children in Ghana were described by Dr D. Candotti (University of Cambridge, UK). In Ghana, approximately 8% of children have anti-B19V IgG, rising to 80% in adults. Viral loads and levels of anti-B19V IgM are higher in children. The rate of persistent infection is ~1·4%. One of the most striking observations of B19V infection in Ghana is that the circulating viruses are almost all genotype 3 [19]. DNA sequence analysis has revealed that the genotype 3 viruses can be divided into two subtypes or clusters that differ by more than 5% nucleotide identity [20]. These have been termed 3a and 3b, and the clustering is independent of the region of the B19V genome analysed. The nucleotide substitution rates were examined for B19V in Ghana and compared with V9, the prototype genotype 3 virus, identified nearly 10 years ago [8]. It was found that like genotype 1 B19V and canine parvoviruses, the genotype 3 viruses have an unexpectedly high rate of evolutionary change [21,22]. It would appear that the type 3a and 3b clusters were derived from a common ancestor approximately 500 years ago; however, there is a wide interval around this date.

Presence of different B19V genotypes in plasma products and susceptibility to inactivation

Professor A.-M. Eis-Hübinger (University of Bonn, Germany) reviewed studies performed on factor VIII and factor IX concentrates to determine the frequency of contamination of these products with genotypes 1 and 2 of B19V. A total of 202 different lots of clotting factor concentrates were examined. Older products used until the early 1980s that had not undergone viral inactivation procedures (21 lots, representing eight different products) were compared with more recent batches in use between 2000 and 2003 (181 lots, representing 13 different products). In the factor VIIIIs, 81% were contaminated with genotype 1 B19V, and 14% were contaminated with genotype 2. In the more recent factor VIIIIs, 46% were contaminated with genotype 1 B19V and 1·6% were contaminated with genotype 2 (two products were co-contaminated with genotype 1). The highest loads of genotype 1 and genotype 2 B19V were ~10⁷ and ~10⁵ geq/ml, respectively. It was suggested that the much lower frequency of detection of genotype 2 B19V is due to generally lower prevalence compared to genotype 1 [23].

Dr M. W. Yu (CBER) described a study looking at factor VIII concentrates using a consensus polymerase chain reaction (PCR) for genotypes 1–3 for B19V, followed by specific restriction endonuclease digestion of the product to discriminate genotype 1 from genotypes 2 and 3. A range of products ($n = 202$ lots) of differing purity produced before 1984 until 2004 were analysed. Of these, 79 lots were positive in the initial screening assay, and a single lot, from 1997, was positive for B19V genotype 2. DNA sequence analysis confirmed the genotype of this virus. This final product did not contain any genotype 1 B19V, and the load for genotype 2 was 10³ geq/ml. In the case of more recent lots, contamination with B19V was generally less frequent, reflecting the implementation of NAT screening by the manufacturers [24].

Dr M. Nübling (Paul Ehrlich Institute; PEI, Langen, Germany) presented data on behalf of Dr J. Blümel (PEI) comparing the biological and physicochemical properties of B19V genotypes 1 and 2 (isolate IM-81) [25]. Infection of the cell line KU812Ep6 with the two B19V genotypes revealed that there were no differences in expression of the capsid at either the mRNA or protein level. Thermal inactivation occurs through the disintegration of the capsid proteins, with no differences observed between the two genotypes. Virions were heated and subjected to DNase treatment prior to Southern blotting to analyse the integrity of the viral genomic DNA. Treatment of 5% albumin solution, spiked with B19V and heated to 56 °C, resulted in the same temporal inactivation kinetics, regardless of virus genotype. Similar inactivation profiles were observed for genotype 2 B19V, as had been shown previously for genotype 1 B19V virus, when subjected to low pH conditions [26].

Professor Jean-Pierre Allain (Cambridge) outlined a PCR inhibition method, utilizing a preamplification step to quantify B19V inactivation by photochemical treatment using amotosalen (S59). This molecular approach to measuring the inactivation effects of S59 on B19V has been established as an alternative to *in vitro* culture of the virus [27].

Experience with commercial and in-house assays for the detection and quantification of B19V DNA

The performance of two commercially available kits for the quantification of B19V DNA was reviewed by Dr S. Baylis (NIBSC, UK). The first kit, the Roche parvovirus B19 quantification kit for the LightCycler, only detects genotype 1 B19V. When equivalent copy number (10⁶) were analysed for the three genotypes, no amplification plots were observed for genotypes 2 and 3 B19Vs in this real-time assay. However, analysis of amplification products by gel electrophoresis revealed that all three genotypes were amplified, with a much reduced signal for genotype 2 B19V, suggesting mismatches in primer and probe sequences. In the case of the Artus RealArt Parvo

LC kit, good amplification plots were observed for genotypes 1, 2 and 3a of B19V, while the genotype 3b virus was under quantified by approximately 2–3 logs generating much later threshold cycle (C_t) values, which could have an impact on the threshold concentration of 10 IU/ml applying to certain plasma pools [28–30]. Primer and probe sequences are of critical importance in the detection of variant viruses, this is further complicated with requirements to perform quantitative assays.

Dr T. Cuypers (Sanquin, Amsterdam, the Netherlands) described the experience of running two assays concurrently for B19V in a screening centre. The assays included the commercially available Roche LightCycler assay and a previously published consensus assay [28], validated in-house. Screening assays, performed during the previous 2 years, identified three instances where discrepant results occurred between the two tests. Molecular characterization was performed to identify the reasons for the discrepant results. One sample, not detected in the Roche assay was found to be a genotype 2 B19V, containing mutations in the primer and probe binding regions. A genotype 1 sample was under quantified by $\sim 2 \log_{10}$ in the Roche assay compared with the in-house assay, with a mutation at or near the end of the reverse primer binding region in the Roche test. In a third case, there was a single point mutation in the probe binding site of the in-house assay, which resulted in a failure to detect a genotype 1 B19V in the plasma sample [31]. Genotypes 2 and 3 for B19V appear to be very rare in Dutch and Belgian donors.

Dr Marta José (Grifols, Barcelona, Spain) described the validation of both in-house qualitative and quantitative consensus B19V assays, for the detection of all three genotypes. Validation was performed according to current guidelines. Particular attention was paid to B19V assay specificity, with no cross-reactivity observed with other blood borne viruses. A variety of genotype 2 and genotype 3 B19V-positive plasma samples were analysed, and good correlations were found with previously determined titres from other laboratories and the ones determined by the in-house quantitative assays.

Dr T. Gierman (Talecris, Raleigh, NC, USA) was unable to attend the meeting and his presentation on experience in testing for B19V genotypes was summarized by Dr Zerlauth.¹

¹Dr G. Zerlauth (Baxter, Vienna, Austria) summarized this special meeting at SoGAT XX, held in Warsaw, Poland, on 12–13 June 2007. At the same meeting, Drs L. Rinckel and T. Gierman (Talecris, Raleigh, NC, USA) reported that they have identified a high-titre, high-volume genotype 3 B19V plasma. Thus, in order to harmonize results obtained by control laboratories and plasma fractionators, a genotype panel containing each of the three genotypes of B19V will be jointly formulated by NIBSC and CBER. The panel will be evaluated together with additional genotype 2 samples and be calibrated against the current WHO International Standard for B19V DNA (99/800) in an international collaborative study. The presentations from the extraordinary SoGAT meeting are available at the following link: http://www.nibsc.ac.uk/partners/SoGAT/March_2007_Presentations.html.

Three tests are utilized to reduce B19V viral loads in plasma fractionation pools: a qualitative donor sample test for testing mini-pools; a separate qualitative test for the QC of fractionation pools; and a quantitative test using dual-labelled fluorogenic detection probes for quality and technical operations investigations. As part of on-going efforts to assess the performance of this test system, the potential frequency of occurrence of variant B19V genotypes in US source plasma was examined. Archived sample pools created from 'non-elevated' plasma samples (samples containing B19V genotype 1 titres $> 2 \times 10^5$ IU/ml and genotypes 2 and 3 titres $> 5 \times 10^6$ copies/ml would have been excluded as a result of screening with the donor sample test) were retested using a fluorogenic detection probe capable of differentially detecting B19V genotypes 2 or 3. The testing of 242 large-scale sample pools (3840 samples) and 609 intermediate-scale pools (960 samples) failed to identify the presence of B19V genotypes 2 and 3. PCR analysis of 340 individual 'elevated' samples also failed to identify B19V genotypes 2 or 3 among them. The inability to detect B19V genotypes 2 and 3 in material representing approximately 1.5 million source plasma donations, which suggests that the prevalence of these genotypes within the US source plasma donor population is very low.

Provision of plasmid clones to OMCL network and manufacturers

Dr J. Fryer (NIBSC, UK) discussed how plasmid clones representing the main B19V genotypes would be distributed through the next EDQM PTS for B19V DNA later in 2007. High-titre DNA stocks have been prepared for near full-length plasmid clones, representing genotypes 1, 2, 3a and 3b of B19V (N8, A6, V9 and D91-1, respectively). Dilution to equal copy number gave equivalent results using a consensus in-house TaqMan assay for B19V DNA [28]. These plasmid clones will be distributed as a validation panel only for use in the PTS, until a plasma reference panel becomes available.

Availability of B19V viraemic plasma for reference panel development

Genotype 2

Several plasma units were identified that contained high titres of genotype 2 B19V. Dr M. Gessner (Baxter, Vienna, Austria) described the B19V plasma samples termed IM-81 and IM-82. IM-81 was a high-titre ($\sim 11.3 \log_{10}$ IU/ml) genotype 2 plasma sample, which had been sequenced and characterized previously [25] and shown to be cross-neutralized by genotype 1 sera. IM-82 represents a subsequent bleed (4 days later) from the same donor with a titre of $7.4 \log_{10}$ IU/ml. Dr S. Baylis (NIBSC) described a plasma pool, sourced in the

USA containing a genotype 2 B19V. The pool was identified due to discrepant results, using different B19V NAT assays. This pool contains $6.2 \log_{10}$ IU/ml of genotype 2 B19V DNA. The virus was not infectious in culture, and was likely to be neutralized by anti-B19V present in the pool. Despite the plasma being pooled, there was no genotype 1 B19V present. Dr M. Koppelman (Sanquin, Amsterdam, the Netherlands) described the identification of a genotype 2 B19V plasma sample (207458), with a titre of $\sim 7 \log_{10}$ IU/ml. Sequence analysis indicated that this B19V was most closely related to the A6 virus [31]. Dr José (Grifols) described another genotype 2 B19V plasma sample. This sample was identified by using two different assays: the first specific for genotype 1 B19V and the second, a consensus assay described in her earlier presentation. The plasma gave negative results in the genotype 1 B19V assay, but was positive in the consensus assay. This led to further characterization of the plasma sample, which was found to have a titre of $7.3 \log_{10}$ IU/ml. The sample was negative for a range of other virus markers, and was also negative for anti-B19V IgG and IgM, and likely to represent the early ramp-up phase.

Genotype 3

While several high-titre, high-volume plasma samples have been identified for genotype 2 B19V, there is limited material available for genotype 3. Dr D. Candotti (University of Cambridge) summarized a series of clinical samples, comprising both genotypes 3a and 3b viruses. None of the available samples exceeded $\sim 6 \log_{10}$ IU/ml of B19V DNA. It was proposed that B19V samples might be sought prospectively, by identifying persistent infections in blood donors (which may have titres as high as $4-5 \log_{10}$ IU/ml). However, several thousand donations would have to be screened. Additional sources of genotypes 2 and 3 B19V have been examined and these include the screening of anti-B19V IgM-positive sera from Brazilian patients presenting with rash-like illness by Dr K. Brown (HPA). In a recently published study from Brazil [32], clinical samples from patients with B19V-like symptoms were tested for B19V DNA and the virus genotype determined. All three genotypes of B19V were identified. Dr K. Brown outlined the approach taken in his study using biotinylated PCR products and pyrosequencing to determine the genotype of each B19V-positive sample. The method was validated using previously identified variant viruses [30]. Of 50 B19V IgM-positive samples studied by this approach, 29 were positive for B19V DNA by PCR, ranging in concentration from 10^2 to 10^{10} geq/ml. These PCR-positive viruses were all genotype 1, with three unique point mutations being identified. A small study was presented by Dr S. Baylis on behalf of Dr D. York (Molecular Diagnostic Services Pty Ltd, South Africa) and Mr D. Stubbings (National Bioproducts Institute, South Africa). High-titre B19V plasma donations ($n = 9$)

were genotyped and in contrast to the findings on the West Coast of Africa, these B19V-positive samples were all genotype 1.

Conclusions and recommendations

Overall, based upon the classification by the ICTV and in terms of what is currently known about the biological and serological properties of the different genotypes of B19V, these genotypes clearly represent strains of the same virus. The more recently identified variants appear not to be so well represented in Europe and North America as genotype 1 B19V. However, different genotypes of B19V have been found in donor plasma that has led to batch release issues and based upon recent PTS studies, some assays have proved ineffective in detecting genotype 2 B19V DNA [7]. In order to harmonize the results obtained for the detection and quantification of B19V DNA between control laboratories and the manufacturers of plasma derivatives, it was agreed that standardization of assays using well-characterized reference materials would be the way forward.

The consensus opinion at the meeting was to produce a genotype panel of plasma samples representing the different genotypes of B19V. As B19V DNA testing has a quantitative limit (10 IU/ml), any reference panel would be required to reflect the need for accuracy around this threshold concentration. Future collaborative studies used to evaluate candidate plasma samples for a reference panel would need to be calibrated against the WHO International Standard for B19V DNA [4]. In the absence of sufficient genotype 3 B19V material, it was felt that cloned DNAs may be suitable for preparing a panel, until a plasma reference panel becomes available. The European common technical specifications for in vitro diagnostic medical devices permits the use of materials such as cloned DNAs (independently quantified by spectrophotometry) where a suitable source of native material is absent. What was emphasized throughout the meeting is the importance of depositing DNA sequence for B19V strains in the databases, to ensure that as much information is available as possible to enable good assay design. However, genetic variation was to be expected in the future, including genotype 1 B19V variants, and robust assay design is essential to deal with inevitable genetic changes.

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Appendix

The meeting participants were from regulatory/research/reference laboratories, kit manufacturers, and plasma derivative manufacturers. The following is a list of speakers at the meeting: Dr G. Zerlauth, Baxter, Austria; Dr K. Brown, HPA, UK; Dr J.-M. Spieser, EDQM, France; Dr M. W. Yu, CBER, Bethesda, MD, USA; Ms K. Hokynar, Haartman Institute, Finland; Professor S. Modrow, University of

Regensburg, Germany; Dr D. Candotti, University of Cambridge, UK; Professor A.-M. Eis-Hübinger, University of Bonn, Germany; Dr M. Nübling, Paul Ehrlich Institute, Germany; Professor J.-P. Allain, University of Cambridge, UK; Dr S. Baylis, NIBSC, UK; Dr T. Cuijpers, Sanquin, the Netherlands; Dr M. José, Grifols, Barcelona, Spain; Dr J. Fryer, NIBSC, UK; Dr M. Gessner, Baxter, Austria; Dr M. Koppelman, Sanquin, the Netherlands. The meeting was chaired by Dr P. Minor (NIBSC, UK).

