Neutralization of human parvovirus B19 by plasma and intravenous immunoglobulins

Jens Modrof, Andreas Berting, Björn Tille, Andreas Klotz, Christina Forstner, Sandra Rieger, Claudia Aberham, Matthias Gessner, and Thomas R. Kreil

BACKGROUND: Human parvovirus B19 (B19V) is a highly prevalent pathogen, and plasma pools for manufacturing of plasma-derived products have been shown to contain antibodies against B19V (B19V immunoglobulin G [IgG]).

STUDY DESIGN AND METHODS: The megakaryoblastic cell line UT7/Epo-S1 can be infected with B19V Genotype 1 and as demonstrated here by immunocytochemistry, Western blot, and reverse transcription–polymerase chain reaction (RT-PCR) of B19V-specific mRNA, also with the more recently discovered Genotype 2. Based on B19V RT-PCR analysis of infected UT7/Epo-S1 cells, an infectivity assay was established and implemented for a B19V neutralization assay. To investigate the role of B19V neutralization in relation to B19V IgG titers, more than 1000 manufacturing plasma pools were tested by enzyme-linked immunosorbent assay.

RESULTS: Plasma pools were found to contain a mean B19V IgG titer of 33 ± 9 IU per mL, with the lowest titer at 11 IU per mL. These 11 IU per mL B19V IgG neutralized 4.6 log B19V Genotype 1 and greater than 3.9 log Genotype 2 infectivity. Accordingly, a 10 percent intravenous immunoglobulin (IVIG) product prepared from such pools was found to contain an even higher B19V neutralization capacity.

CONCLUSION: A high capacity of B19V Genotypes 1 and 2 neutralization was demonstrated in plasma pools for fractionation, an inherent feature based on the constantly high titer of B19V IgG in these pools. The neutralizing activity of B19V IgG was shown to be maintained in the 10 percent IVIG product tested.

Human parvovirus B19 (B19V) belongs to the genus *Erythrovirus* (family *Parvoviridae*), which has recently been reclassified to contain three different B19V genotypes (1-3).[1] Although B19V Genotype 1 is by far the most prevalent, Genotype 2 has also been sporadically detected in Europe and was shown to occur in plasma pools for manufacturing into plasma derivatives.[2,3] B19V Genotype 3 appears to be mostly restricted to West Africa.[4]

Soon after its initial identification,[5] B19V was recognized to cause fifth disease in children (erythema infectiosum), whereas more serious clinical manifestations of B19V infection were only recently understood to include arthropathy, transient aplastic crises, persistent anemia, and hydrops fetalis.[6] In addition, an association of B19V with inflammatory heart disease in adults has lately been suggested.[7,8] The only treatment option available for B19V infection so far is intravenous immunoglobulin (IVIG), although based on anecdotal evidence rather than, for example, established dose–response correlations.[9]

Owing to the global prevalence of B19V Genotype 1, 30 to 60 percent of adults carry antibodies against B19V (B19V immunoglobulin G [IgG]), with a good correlation between antibody prevalence and age.[10] Consequently, the presence of B19V IgG was found in all of the few plasma pools for manufacturing so far investigated.[11-14] Mostly due to the lack of a widely available B19V

ABBREVIATIONS: B19V = parvovirus B19; MOI = multiplicity of infection; NC_{50} = 50 percent B19V neutralization capacity; $TCID_{50}$ = 50 percent tissue culture–infectious dose.

From the Global Pathogen Safety and Plasma Control, Baxter BioScience, Vienna, Austria.

Address reprint requests to: Thomas R. Kreil, PhD, Baxter BioScience, Benatzkygasse 2-6, A-1220 Vienna, Austria; e-mail: thomas_kreil@baxter.com.

Received for publication May 2, 2007; revision received June 21, 2007, and accepted June 26, 2007.

doi: 10.1111/j.1537-2995.2007.01503.x.

TRANSFUSION 2008;48:178-186.

infectivity assay, however, no information was available with respect to antibody function, that is, B19V neutralization, and whether this potentially clinically relevant variable would correlate with the presence of B19V antibodies detected by, for example, enzyme-linked immunosorbent assay (ELISA).

Although still to date a continuous cell culture system that would allow detection of B19V infectivity by determination of a classical cytopathic effect does not exist, B19V replication has been demonstrated in a few specialized cell lines mostly utilizing the detection of de novo transcribed B19V-specific mRNA after infection of and virus replication in these cells.[15-19] Particularly the erythroid progenitor cell line KU812Ep6 was used to detect B19V infectivity and was found to be sensitive to both B19V Genotypes 1 and 2.[18,20] These cells are, however, not widely available. Alternatively, use of the megakaryoblastic cell line UT7/Epo-S1 has also allowed accurate quantification of B19V Genotype 1 infectivity over several orders of magnitude.[15,16,21]

With an internally controlled reverse transcription–polymerase chain reaction (RT-PCR) system (TaqMan, Applied Biosystems, Foster City, CA), an infectivity assay for both B19V Genotypes 1 and 2 in UT7/Epo-S1 cells was established. The presence of B19V IgG antibodies in plasma pools for further manufacturing was confirmed, and the titers of B19V IgG were quantified. With our infectivity assay, a correlation between B19V antibody presence as determined by ELISA and function as determined by neutralization of B19V infectivity was established for manufacturing plasma pools. Given the clinical relevance of B19V antibodies in IVIG as the only treatment option for human B19V infection, particularly given the recent recognition of more severe disease associations[7,8] and emerging B19V variants,[22] the level of B19V IgG function was also determined for a commercially available IVIG preparation.

MATERIALS AND METHODS

Cells, B19V, and plasma

UT7/Epo-S1 cells were provided by K.E. Brown (Health Protection Agency, London, UK; with permission of K. Sugamura, Tohoku University, Sendai, Japan). Cells were maintained in Iscove's modified Dulbecco's medium, containing 10 percent fetal calf serum (JRH Biosciences, Lenexa, CA), 1 percent L-glutamine, 1 percent gentamicin sulfate, and 2 IU per mL erythropoietin (Janssen-Cilag, Neuss, Germany) at 37°C with 5 percent CO_2.

Plasma donations containing high titers of B19V as detected by the routine B19V PCR donor screening program of Baxter BioScience (Plasma Analytics Department, Vienna, Austria) were used as the source of infectious B19V. Plasma Donation 990237 contained B19V Genotype 1 (titer, 11.8 log IU/mL) and Donation IM 81 contained B19V Genotype 2 (titer, 11.4 log IU/mL).

Infection of UT7/Epo-S1 cells with B19V and isolation of mRNA

UT7/Epo-S1 cells (10^5 per six-well) were infected with B19V at multiplicity of infection (MOI) of 10^{-3} to 10^6 and incubated for 7 days or mock infected with buffer for negative controls. Seven days after infection, mRNA was extracted with a direct mRNA miniprep kit (GenElute, Sigma-Aldrich, Vienna, Austria) according to the manufacturer's protocol.

Immunocytochemical staining and Western blot of B19V capsid proteins

For immunocytochemical staining, infected cells were pelleted and fixed on glass slides coated with 70 to 150 kDa poly-L-lysine (0.01 mg/mL in phosphate-buffered saline [PBS]) with 4 percent paraformaldehyde. After permeabilization with 0.5 percent Triton X-100, cells were incubated for 1 hour with a monoclonal antibody (10 μg/mL) specific for B19V capsid proteins VP1 and VP2 (R92F6/MAB8293, against amino acids 328-344 of VP2; Chemicon, Chandlers Ford, UK).[23] After washing (PBS), an anti-mouse–horseradish peroxidase polymer conjugate (SuperPicTure polymer detection kit, Zymed, Vienna, Austria) was applied, and the signal was developed by 15-minute incubation with 3,3′-diaminobenzidine chromogen solution before mounting slides with aqueous mounting medium. To determine the intracellular localization of B19V structural proteins, cells were counterstained with methyl green (Vector, Burlingame, CA) according to the supplier's instructions.

For Western blotting, approximately $10^{5.6}$ cells were collected, 2 to 7 days after infection and washed in Tris-buffered saline (TBS). Proteins were then separated by Bis-Tris sodium dodecyl sulfate (SDS)–4 to 12 percent polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes with a "semidry" discontinuous protein transfer chamber (Immobilon P, Bio-Rad, Munich, Germany). After blocking, membranes were incubated with the primary antibody R92F6 diluted 1:2000. Membranes were washed in 0.5× TBS containing (vol/vol) 0.2 percent Triton X-100 and incubated for 1 hour with polyclonal rabbit α-mouse immune globulins–alkaline phosphatase (at 1:5000; Dako, Glostrup, Denmark). Bound antibodies were detected with a color reaction (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution, Sigma-Aldrich).

RT-PCR of spliced B19V transcripts

Real-time RT-PCR (TaqMan) was performed with a sequence detection system (ABI Prism 7900HT PE Applied

Biosystems) with a RT-PCR core reagent kit (TaqMan EZ, PE Applied Biosystems). The B19V mRNA primers amplify a region from nucleotide position nucleotide 365 to nucleotide 1978, spanning splice donor site nucleotide 406 to splice acceptor site nucleotide 1910 (reference sequence PVBAUA, NCBI GeneBank, Accession Number M13178), that is, effectively preventing B19V DNA amplification by use of an intron-spanning probe. As control for mRNA quality and to exclude false-negative B19V results, all samples were tested in parallel for the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with a predeveloped TaqMan assay reagents human GAPDH kit (PE Applied Biosystems).

Amplification of B19V mRNA was performed with 640 nmol per L each primer (PA3F, 5'-TTTCCTGGAC TTTCTTGCTGTT-3'; PA3R, 5'-CACCACCACTGCTGCTGA TACT-3'), 160 nmol per L the intron-spanning probe (PA3P, 5'-VIC-TTTGTGAGCTAACTAACAGATGCCCTCCAC CCAGAC-TAMRA-3'), and 10 ng of mRNA. Appropriate reagent controls and 200 copies of in vitro transcribed control RNA (10 copies/μL) in duplicate were included on each microtiter plate. Primers and probe for B19V PCR (synthesized by Ingenetix, Vienna, Austria) were high-performance liquid chromatography purified to a purity of greater than 90 percent.

The amplification program for GAPDH and B19V was composed of reverse transcription of mRNA into cDNA (50°C for 2 min and 60°C for 20 min) and subsequent PCR amplification with an initial denaturation step at 95°C for 5 minutes and 45 cycles of denaturation at 94°C for 20 seconds and annealing and extension at 57°C for 1 minute. Analysis of data generated was performed with the TaqMan software (SDS software Version 2.0, PE Applied Biosystems) with a threshold of 0.07 ΔRn fluorescent signal (label, VIC).

B19V neutralization with plasma pools or IVIG

To investigate B19V neutralization at B19V IgG titers of 11 IU per mL or below, two single plasma donations that had been tested nonreactive for B19V IgG were blended with plasma from manufacturing pools (cryorich plasma) to obtain defined titers between 0.4 and 11 IU per mL B19V IgG. The actual B19V IgG titers of these plasma pool blends (4 and 11 IU/mL) were confirmed by ELISA (Novagnost Parvovirus B19 IgG-ELISA; NovaTec, Dietzenbach, Germany).

A quantity of 450 μL of plasma pool blends or original plasma pool samples (cryorich plasma) were mixed with 50 μL of cell culture medium containing between 10^2 and 10^{10} IU B19V. After incubation for 1 hour at 37°C, the entire 500-μL mixture was incubated on 10^5 UT7/Epo-S1 cells for 7 days, before testing B19V infectivity by RT-PCR as described above. To control for any potential complement influence, neutralization experiments were also performed after incubation of B19V IgG containing plasma samples at 56°C for 30 minutes before use. As a control, B19V titers between 10^2 and 10^{10} IU were incubated with the two single plasma donations (both tested nonreactive for B19V IgG and B19V) instead of the B19V IgG containing plasma pool blends or original plasma pools for each experiment.

For each B19V IgG concentration used for neutralization tests at least four (0.4, 4, and 11 IU/mL) or two (1 and 36 IU/mL) independent titrations were performed. A human IVIG product (for Europe, KIOVIG [Baxter Healthcare Corporation, Westlake Village, CA]; for the United States, Gammagard liquid [Baxter Healthcare Corporation]) was used in neutralization tests as described. Because the original IVIG preparation had a B19V IgG titer of 562 IU per mL (manufactured from plasma pools with a mean B19V IgG titer of 40 IU/mL), dilutions ranging in B19V IgG titer from 0.1 to 25 IU were prepared in 0.2 mol per L glycine, the medium of the IVIG preparation.

The results obtained by duplicate TaqMan RT-PCR runs for each sample were analyzed qualitatively, that is, crossing of the TaqMan RT-PCR threshold of 0.07 ΔRn VIC was scored positive while not exceeding that background threshold was scored negative.

Statistical analysis

Statistical evaluation of B19V IgG titer frequencies in manufacturing plasma pools (containing a few thousand donations each), the calculation of B19V titers that equaled 50 percent infectivity and unpaired t tests were calculated with computer software (GraphPad Prism 4, GraphPad Software, San Diego, CA).

RESULTS

Infection of UT7/Epo-S1 cells with B19V Genotype 1 and 2

Although it had been shown that B19V Genotype 1 infectivity can be quantified by RT-PCR analysis of mRNA isolated from infected UT7/Epo-S1 cells, it was suggested that this same assay might not work for B19V Genotype 2.[22] To reinvestigate the susceptibility of UT7/Epo-S1 cells for infection with B19V Genotype 2, cells were in parallel exposed to either B19V Genotype 1 or B19V Genotype 2, and the following events were examined by immunocytochemical staining and Western blot (Fig. 1).

Immunocytochemistry of cells prepared on Day 1 after B19V infection served as negative control that confirmed that inoculum virus was sufficiently removed. Cells prepared on Day 3 after infection, however, revealed the presence of newly synthesized B19V capsid proteins within infected UT7/Epo-S1 cells, after exposure to both B19V Genotype 1 or B19V Genotype 2 (Fig. 1A). As sug-

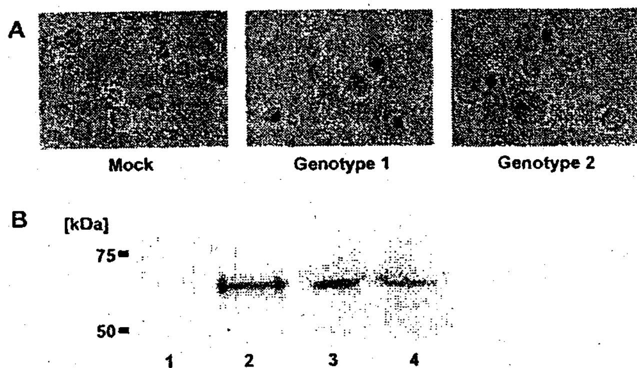


Fig. 1. Infection of UT7/Epo-S1 cells with B19V Genotypes 1 and 2. (A) Immunocytochemical staining of UT7/Epo-S1 cells that were mock-infected, infected with B19V Genotype 1 (MOI, 10^{6}) or B19V Genotype 2 (MOI, $10^{5.6}$) for 7 days. Original magnification, ×40. (B) Western blot of cell lysates from UT7/Epo-S1 cells. Lane 1 = negative control, mock-infected cells; Lanes 2 through 4 = B19V-infected cells; Lane 2 = Genotype 1 (MOI, 10^{5}); Lane 3 = Genotype 1 (MOI, 10^{4}); Lane 4 = Genotype 2 (MOI, 10^{5}).

gested by Fig. 1A, and confirmed by counterstaining of infected cells with methyl green (not shown), the VP1/VP2 staining was primarily confined to the nucleus. Quantitatively, approximately 10 percent cells infected with either Genotype 1 or Genotype 2 were positive on Day 3 after infection. Seven days after infection UT7/Epo-S1 cells had multiplied to an approximately 10-fold higher cell number, yet the percentage of infected cells was still at approximately 10 percent (data not shown).

The detection of B19V capsid proteins by Western blot also confirmed the susceptibility of UT7/Epo-S1 cells to infection with both B19V Genotype 1 and B19V Genotype 2 (Fig. 1B). The predominant B19V capsid protein splice variant VP2 was clearly detectable for both B19V genotypes at 7 days postinfection, although the signal for Genotype 1 appeared somewhat stronger compared to the signal for Genotype 2. This effect became more apparent when the MOI used for Genotype 1 was 10 times lower than that for Genotype 2 (Fig. 1B, Lanes 3 and 4). Cell homogenates obtained during Days 1 through 4 after infection did not result in detectable Western blot signals, confirming de novo synthesis of B19V proteins rather than detection of residual inoculum virus particles. The detection limits of B19V infectivity determined by immunocytochemical staining and Western blot were for both approximately 10^{9} IU B19V.

The presence of B19V proteins in infected UT7/Epo-S1 cells as now demonstrated for both B19V Genotype 1 and B19V Genotype 2 would, as a prerequisite, require production of B19V-specific mRNAs, spliced exactly as during infection of humans.[24] The presence of these spliced mRNA species would then provide the basis for detection of B19V infectivity by TaqMan RT-PCR as described under Material and Methods.

As expected, RT-PCR confirmed infectivity of B19V Genotypes 1 and 2 for UT7/Epo-S1 cells. For an accurate calculation of the B19V PCR titer that corresponds to one 50 percent tissue culture–infectious dose ($TCID_{50}$), at least eight replicates of the B19V titers that did not result in all negative or in all positive RT-PCR results were analyzed (Table 1). The respective $TCID_{50}$ values obtained were 3.7 log IU for B19V Genotype 1 versus 6.1 log IU for B19V Genotype 2.

Neutralization of B19V

Plasma manufacturing pools consist of typically several thousand individual donations and at a B19V IgG seroprevalence of approximately 30 to 60 percent in the plasma-donating population[10] statistically rather evenly distributed B19V antibody levels should be expected to occur in plasma pools. To establish a statistically meaningful estimate of the B19V antibody concentrations present in plasma manufacturing pools, samples were obtained from a total of 1174 pools, representing a few million donations collected in either Europe or the United States over the course of 2 years, to be tested for B19V antibodies by ELISA.

As expected from the high seroprevalence of B19V in the human population, the analysis revealed a rather high mean ELISA titer of 33 ± 9 IU per mL (mean ± SD) among all the plasma pools tested, with a high of 71 IU per mL and a low of 11 IU per mL (Fig. 2A). Although thus the presence of a varying yet significant level of B19V antibodies had been confirmed for a statistically relevant number of plasma pools by ELISA, we sought to measure antibody function—rather than presence—considering this clinically more meaningful.

Neutralization of B19V infectivity in plasma pool specimens was thus investigated, utilizing the detection of B19V infectivity by TaqMan RT-PCR, after incubation in the presence or absence of defined concentrations of B19V IgG as determined by ELISA. Of potentially the most significant clinical importance, the B19V neutralization capacity at the lowest ELISA titer ever found for a plasma manufacturing pool in our investigation, that is, 11 IU per mL, was investigated (Fig. 2A). The test material was derived by blending plasma manufacturing pool specimens of higher B19V antibody titer (36-40 IU/mL B19V IgG) with plasma donations individually screened to be nonreactive for B19V IgG and B19V, to reach the target concentration of 11 IU per mL B19V IgG.

The comparison of B19V Genotype 1 infectivity after incubation with either B19V antibody–negative plasma (control) or the plasma pool sample blended to contain 11 IU per mL B19V IgG can be seen in Fig. 2B. For each data point given in Fig. 2B, at least five infectivity tests

TABLE 1. Infectivity of B19V Genotypes 1 and 2, detected by mRNA TaqMan RT-PCR*

B19V (IU) for infection	Detection of B19V infectivity by mRNA RT-PCR	
	Genotype 1	Genotype 2
10^{10}	2+	8+
10^{9}	2+	12+
10^{8}	2+	7+/1–
10^{7}	2+	10+/2–
10^{6}	18+	5+/5–
10^{5}	12+	6–
10^{4}	9+/3–	NT
10^{3}	1+/9–	NT
10^{2}	10–	NT
$TCID_{50}$ (log)	3.7	6.1

* Seven days after infection of UT7/Epo-S1 cells with different B19V concentrations, mRNA was isolated and subjected to TaqMan RT-PCR. From the numbers of positive (+) and negative (–) results obtained at certain virus dilutions, the virus titer corresponding to 50 percent infectivity ($TCID_{50}$) was calculated.
NT = not tested.

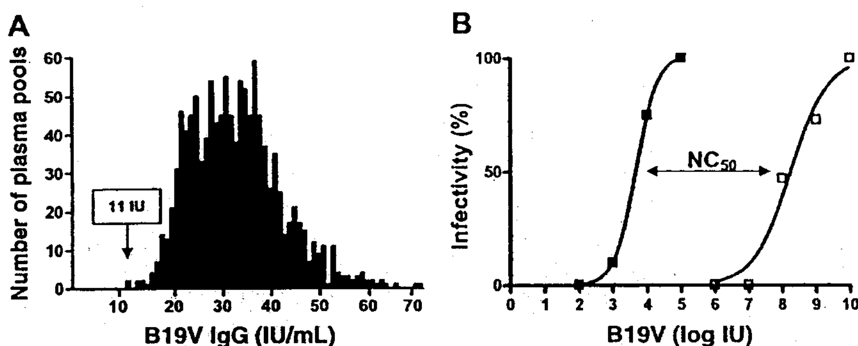


Fig. 2. Neutralization of B19V by plasma manufacturing pools. (A) B19V IgG antibody titers of 1174 plasma manufacturing pools as determined by ELISA (IU/mL). (B) Infectivity (%, detected by TaqMan mRNA RT-PCR) of B19V samples on UT7/Epo-S1 cells that were inoculated with 10^2 to 10^6 IU B19V (■) or 10^6 to 10^{10} IU B19V incubated with 11 IU per mL B19V IgG before infection of the cells (□). The difference in B19V concentrations that corresponds to 50 percent B19V infectivity between the two sets of samples represents the NC_{50} of B19V IgG at 11 IU per mL.

have been performed to determine the percentage of infectivity from the respective number of positive and negative TaqMan RT-PCR results. Compared to the $TCID_{50}$ of 3.7 log IU per mL for B19V Genotype 1 in the presence of control plasma, incubation with the plasma containing 11 IU per mL B19V IgG increased the $TCID_{50}$ to a corresponding B19V concentration of 8.2 log IU per mL. From these results, the 50 percent B19V neutralization capacity (NC_{50}) for plasma containing 11 IU per mL B19V IgG could be calculated, that is, the difference of both 50 percent infectivity calculations, as 4.6 log B19V IU per mL.

To substantiate the relevance of this result that was obtained by use of blended plasma specimens, samples from the two original plasma manufacturing pools from 2004 and 2005, each containing only 11 IU B19V IgG per mL, were also investigated in the same neutralization assay setup. Although the available sample volumes from these two specific plasma manufacturing pools were limited, only three neutralization assays could be performed with the original pool samples, demonstrating a mean NC_{50} neutralization capacity of 4.6 log B19V IU per mL, thus confirming the results earlier obtained with the blended plasma samples.

To establish a dose–response relationship between the B19V neutralization capacity and the presence of B19V antibodies as determined by ELISA, samples at B19V IgG concentrations higher than the 11 IU per mL or even lower, that is, concentrations that never occur in plasma manufacturing pools, were generated with the blending approach described. These samples, at B19V antibody concentrations between 0.4 and 36 IU per mL B19V IgG, were subsequently tested for their B19V neutralization capacity (Fig. 3).

The NC_{50} obtained for a plasma sample containing an artificially low 0.4 IU per mL B19V antibody titer was 1.8 log B19V. At 1 and 4 IU per mL B19V IgG, that is, still well below the lowest B19V IgG concentrations ever observed in a plasma manufacturing pool, 3.1 log and 4.4 log B19V IU per mL were neutralized, respectively. At B19V antibody concentrations higher than the earlier tested 11 IU, the demonstrable B19V neutralization did just marginally increase, showing a NC_{50} of 4.9 log B19V at 36 IU per mL B19V IgG.

Neutralization experiments with B19V Genotype 2 were somehow limited by the significantly higher limit of detection for Genotype 2 compared to Genotype 1 (Table 1). Specifically, already at 11 IU per mL B19V IgG the NC_{50} was beyond the limit of detection for the method, and the corresponding result was neutralization of greater than 3.9 log for B19V Genotype 2. This level of neutralization, however, was well comparable to the one earlier observed for B19V Genotype 1.

Because IVIG products are indicated for the treatment of severe B19V infections, information about the functional B19V neutralization capacity of such products would be clinically relevant. Consequently, the neutralization capacity of IVIG samples was tested. Because the original IVIG preparation contained 562 IU per mL B19V IgG (corresponding to 5.62 IU B19V IgG/mg IgG), predilution to contain B19V IgG titers between 0.1 and 25 IU was

required for evaluation by the neutralization assay. Coherent with the results earlier obtained for plasma samples, 2.2 log infectious B19V were already neutralized by an IVIG sample at 0.3 IU B19V antibody, and 5.2 log infectious B19V were neutralized by an IVIG specimen diluted to 8.4 IU B19V IgG.

Anti B19V titers in different manufacturing pools

Comparing the B19V IgG content of different US plasma manufacturing pools (pooled between January 5, 2004, to November 18, 2005), prepared from either source plasma, that is, collected by plasmapheresis, or recovered plasma, that is, derived from whole-blood donations, we found significantly different B19V IgG titers depending on the plasma source: while recovered plasma pools (n = 48) contained a mean ± SEM titer of 43 ± 1.5 IU per mL (range 22-71 IU/mL), source plasma pools (n = 630) only had 31 ± 0.3 IU per mL (range, 11-53 IU/mL). These differences were significant, as shown by analysis of the B19V antibody titers between source and recovered plasma pools by unpaired t test ($p < 0.0001$; Fig. 4A).

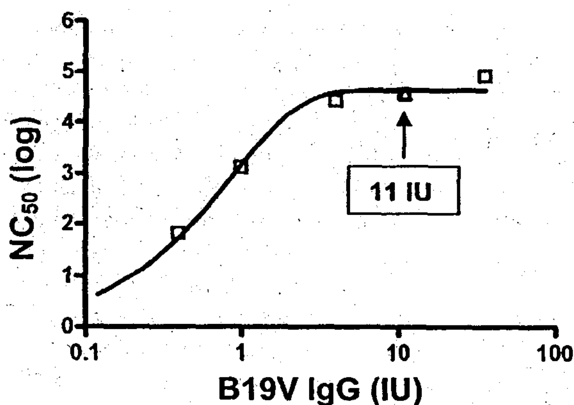


Fig. 3. Dose-response relation for neutralization of B19V. Relation of B19V infectivity NC_{50} (see Fig. 2) and concentrations of B19V IgG (IU).

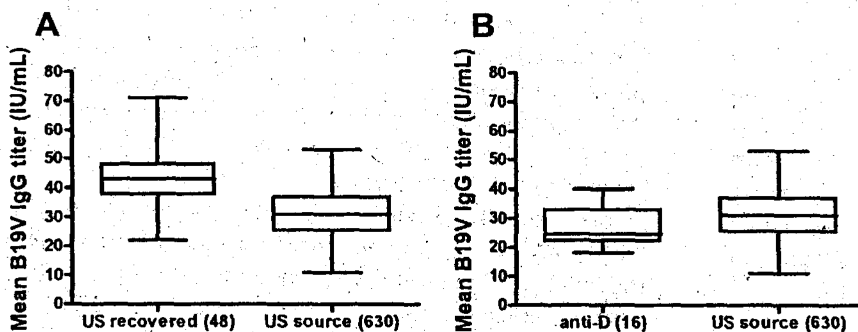


Fig. 4. Comparison of mean B19V IgG titers in plasma manufacturing pools, produced in 2004 and 2005, by unpaired t test. (A) US source (n = 630) versus US recovered (n = 48), $p < 0.0001$. (B) Anti-D (n = 16) versus US source (n = 630), $p = 0.0694$.

Whereas for the plasma pools so far tested for B19V antibodies an industry voluntary standard[25] required limiting the presence of B19V by PCR pretesting to less than 10^5 IU per mL, a limit of less than 10^4 IU per mL B19V has been mandatory for pools of anti-D plasma.[26] Reaching this lower limit requires interdiction of additional donations that contain B19V. As such donations might be expected to also contain B19V antibodies,[6] it is conceivable that the different PCR testing strategies applied for regular versus anti-D plasma might also result in somewhat different B19V IgG concentrations.

Comparing the ELISA B19V IgG results, however, for anti-D plasma (n = 16) and for regular US source plasma (n = 630), that is, plasma types that with respect to B19V only differ in the respective cutoff limits for PCR testing, they were not significantly different ($p = 0.0694$; Fig. 4B).

DISCUSSION

With the emergence of the A6 and V9 erythroviruses,[22,27] that is, viruses that have now been reclassified as B19V genotypes, a useful assay for quantification of B19V infectivity should also be capable of quantifying these genotypes or at least Genotype 1 as the by far most prevalent and Genotype 2 that has been shown to—more rarely—also occur in plasma for fractionation. In contrast to an earlier suggestion that UT7/Epo-S1 cells might not be susceptible to B19V Genotype 2,[22] infection of UT7/Epo-S1 cells by both B19V Genotype 1 and Genotype 2 is demonstrated in the current work. This apparent discrepancy with earlier results is likely due to human immunodeficiency virus coinfection of the earlier used Genotype 2 B19V specimen and its heat inactivation before use in the B19V study, a procedure that possibly would inactivate B19V infectivity.[18] Even in our study, however, the detection of B19V Genotype 2 by Western blot was approximately 10 times less sensitive compared to Genotype 1, and with the TaqMan RT-PCR B19V the limit of detection for Genotype 1 was approximately 100 times lower than for Genotype 2 (Table 1). For the RT-PCR assay, this difference was initially considered to be the consequence of a few mismatches of the primers used for B19V Genotype 2. Another set of primers was therefore designed and tested, specifically designed for the sequence of the B19V Genotype 2 sample used.[20] Even with those, however, the same detection limit was determined as with the Genotype 1 specific primers, suggesting that B19V Genotype 2 infects UT7/Epo-S1 cells somewhat less efficiently compared to B19V Genotype 1, a possible explana-

tion also for the Western blot discrepancy between the two genotypes. Genotype 1 B19V was detected with one $TCID_{50}$ corresponding to 3.7 log IU, that is, well in agreement with results from other investigations.[15,16,28]

Owing to the high prevalence of B19V in the population, the titer of anti-B19V in plasma pools for manufacturing was anticipated to be high and rather constant.[11] Data on B19V IgG in plasma pools available so far, however, were rather limited, with only 20 to 66 plasma pools investigated with mean (± SD) B19V IgG contents ranging from 29.8 ± 17.2 to 64.7 ± 17.5 IU per mL.[11-14] Here a total of 1174 plasma pools were analyzed, representing a few million donations collected over a period of 2 years in both the United States and the European Union, which revealed a mean (± SD) B19V IgG titer of 33 ± 9 IU per mL. Of all these pools, the minimum B19V IgG titer ever found was 11 IU per mL. In the following series of neutralization experiments, correlating the presence of B19V IgG (ELISA) to functional capacity and/or neutralization, B19V Genotype 1 neutralization by B19V IgG was found to be dose-dependent with a neutralization capacity greater than 4 log B19V already at only 4 IU per mL, that is, an artificially low antibody concentration compared to the naturally occurring minimal concentration in plasma pools (11 IU/mL B19V IgG). A neutralization capacity of greater than 4 log has also been shown for higher B19V IgG concentrations, at up to 36 IU per mL. Neutralization of B19V Genotype 2 with 11 IU per mL B19V IgG was likewise shown to be greater than 3.9 log, although a more exact quantification was limited by the higher limit of detection for B19V Genotype 2 in the assay used. In agreement with this, other neutralization experiments with the KU812Ep6 cell line also showed neutralization of more than 4 log B19V infectivity by less than 3.5 IU per mL B19V IgG.[20] In contrast to our study, however, only single plasma donations instead of plasma manufacturing pools were investigated. Even for these individual samples, Blumel and coworkers[20] have indicated B19V antibody cross-reactivity for different B19V genotypes. Based on the contribution of many thousand donors for any plasma manufacturing pool, it can be expected that an even broader range of different B19V genotypes can be neutralized with B19V IgG from plasma pools, a perspective of particular importance for the B19V neutralization capacity of IVIG products produced from these pools. An experimental comparison of the neutralization capacity of plasma manufacturing pools and IVIG showed that with only 8 IU B19V IgG contained within an IVIG sample greater than 5 log B19V were neutralized. A similar degree of B19V neutralization can also be extracted from recent work by another group.[29]

Although several reports have described the transmission of B19V through plasma derived products, the amount of B19V that represents an infectious dose in humans is not yet clear. In one anecdotal B19V transmission episode, however, solvent/detergent–treated plasma that contained $10^{7.5}$ genome equivalents (geq) B19V per mL in the presence of at least 8.8 IU per mL B19V IgG transmitted B19V, while no transmission occurred at a B19V load of $10^{3.5}$ geq per mL or less.[30,31] This observation is in good agreement with our neutralization experiments that have shown neutralization of more than 10^4 IU per mL B19V by even only 4 IU per mL B19V IgG (Fig. 3; note that according to an internal validation, for the B19V Genotype 1–positive donation used in this study, virus quantification in IU is equivalent to geq).

The even higher B19V neutralization capacity as shown for an IVIG product compared to plasma pools would also support the use of IVIG as treatment for fulminant B19V infections, based on the potent neutralization efficacy.[9] To extrapolate our data from in vitro neutralization of B19V by IVIG to the clinical situation, we made a comparison with the amount routinely administered during common variable immune deficiency treatment, that is, 100 to 400 mg immunoglobulins per kilogram of human body weight. With a typical volume of 84.4 mL blood per kilogram of body weight,[32] the IVIG preparation (containing immunoglobulins at 10%) is diluted 1:84 to 1:21 during common variable immune deficiency treatment. Because the IVIG preparation investigated in this study contained 562 IU per mL B19V IgG, the concentration of B19V IgG in the human blood after administration would be 7 to 27 IU per mL. We have shown in vitro neutralization of more than 5 log infective B19V with 8 IU anti-B19V IgG, thus indeed indicating efficient protection from infection with B19V conferred by IVIG.

Evidence exists to suggest that the prevalence of B19V IgG in the population constantly increases with age.[10] In this context, our analysis of B19V IgG titers in plasma pools from recovered and source plasma that revealed a significant difference of mean B19V IgG titers may be caused by a difference in the age structure of plasma donors and blood donors. A B19V nucleic acid test (NAT) limit of less than 10^5 IU per mL B19V in plasma pools has been implemented by manufacturers of plasma derivatives as a voluntary standard some years ago.[25] Since the introduction of that test limit, no B19V transmission by plasma-derived products manufactured according to the standard has been observed, to our knowledge. The recent tendency toward tightening the B19V PCR test limit to less than 10^4 IU per mL, which would require interdicting donations that contain lower levels of B19V but may also contain B19V IgG, carries the possibility of also resulting in lower concentrations of B19V IgG in plasma for fractionation.[6] A direct comparison of plasma pools for which a B19V NAT test limit of 10^4 IU per mL has been implemented by regulatory requirement (anti-D plasma[26]) with plasma pools tested to comply with the Plasma Protein Therapeutics Association voluntary B19V PCR testing standard of less than 10^5 IU per mL (US source plasma)

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.

ACKNOWLEDGMENTS

Don Baker, PhD, is acknowledged for providing unconditional support and strategic vision for the project reported. The contributions of the entire Global Pathogen Safety Team, most notably Bettina York, Claudia Schwarr, Karin Berka, and Elisabeth Pinter (cell culture and virus propagation), and Gerhard Poelsler, PhD, for numerous discussions and scientific advice are acknowledged. Katharina Bledowski, MSc, is recognized for her experimental work on B19V infectivity in UT7/Epo-S1 cells. We thank Gerold Zerlauth, PhD, for generously supporting the PCR-related contributions. We thank the entire Plasma Analytic Team, most notably Angela Netik, PhD, and Esther Asch from the Plasma Analytic/Serology Team for providing the ELISA results.

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
医薬部外品　研究報告　調査報告書
化粧品

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。

BYL-2008-0297
080123
人血清アルブミン

Vox Sanguinis (2007) 93, 208–215

ORIGINAL PAPER

© 2007 The Author(s)
Journal compilation © 2007 Blackwell Publishing Ltd.
DOI: 10.1111/j.1423-0410.2007.00957.x

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.

Received: 16 April 2007, revised 11 June 2007, accepted 16 June 2007, published online 16 August 2007

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]. 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

© 2007 The Author(s)

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).

© 2007 The Author(s)

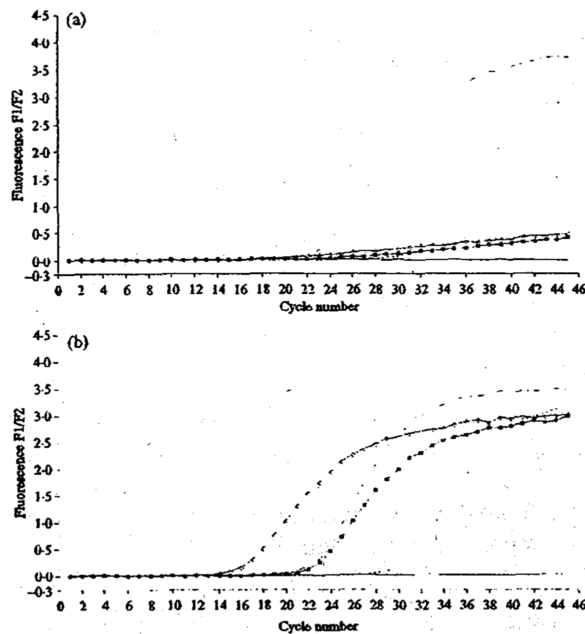


Fig. 1 Amplification plots obtained with the in-house assay under normal conditions using a TaqMan probe labelled with FAM/TAMRA and used at 0·2 μM [5] (a) and the assay performed under normal conditions using a locked nucleic acid (LNA) probe labelled with FAM/BBQ3 and used at 0·02 μM (b).

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

© 2007 The Author(s)

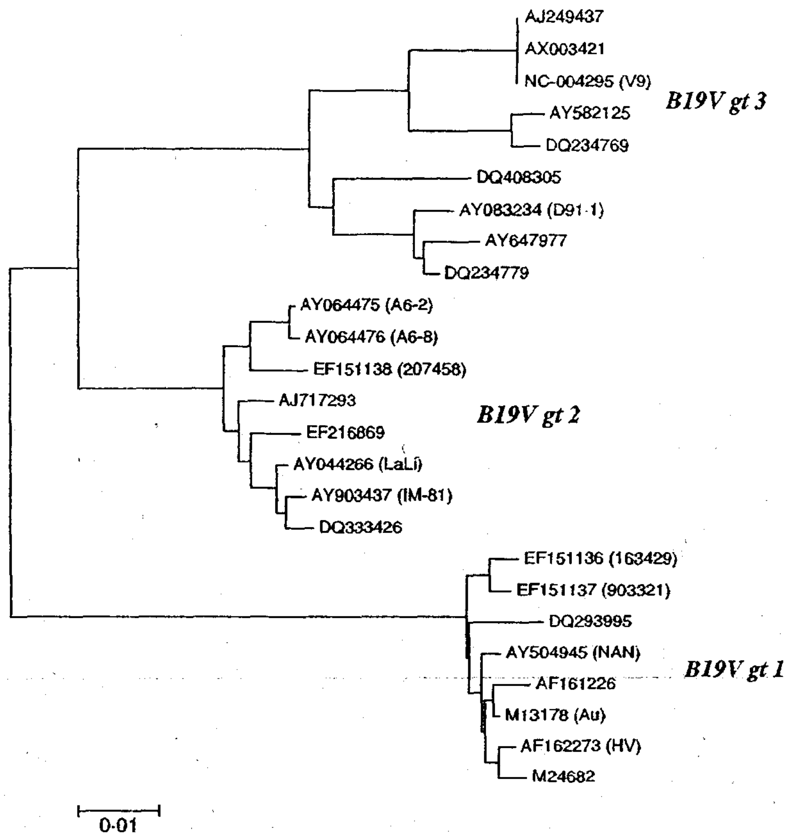


Fig. 2 Phylogenetic tree of parvovirus B19 (B19V) DNA sequences from three B19V variants (903321, 163429 and 207458) and B19V DNA sequences with published genotypes from GenBank. The sequence of a 1536-bp fragment corresponding to part of the NS1–VP1 region was used to create the tree.

(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

© 2007 The Author(s)

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

© 2007 The Author(s)

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.

Acknowledgements

The authors thank Nita Shah from NIBSC, and Mirjam de Waal, Margret Sjerps and Atty Brussee from Sanquin for assistance.

References

1 Anonymous: Human anti-D Immunoglobulin, monograph 0557 (since 1/1/2004); Human anti-D Immunoglobulin for intravenous administration, monograph 1527 (since 1/1/2004); Human plasma (pooled and treated for virus inactivation), monograph 1646 (since 1/7/2004); in *Ph Europe*, 6th edn, Strasbourg, France, Council of Europe, 2006

2 Koppelman MH, Cuypers HT, Emrich T, Zaaijer HL: Quantitative real-time detection of parvovirus B19 DNA in plasma. *Transfusion* 2004; 44:97–103

3 Weimer T, Streichert S, Watson C, Groner A: High-titer screening PCR: a successful strategy for reducing the parvovirus B19 load in plasma pools for fractionation. *Transfusion* 2001; 41:1500–1504

4 Aubin JT, Defer C, Vidaud M, Maniez MM, Flan B: Large-scale screening for human parvovirus B19 DNA by PCR: application to the quality control of plasma for fractionation. *Vox Sang* 2000; 78:7–12

5 Baylis SA, Shah N, Minor PD: Evaluation of different assays for the detection of parvovirus B19 DNA in human plasma. *J Virol Methods* 2004; 121:7–16

6 Aberham C, Pendl C, Gross P, Zerlauth G, Gessner M: A quantitative, internally controlled real-time PCR Assay for the detection of parvovirus B19 DNA. *J Virol Methods* 2001; 92:183–191

7 Nguyen QT, Wong S, Heegaard ED, Brown KE: Identification and characterization of a second novel human erythrovirus variant, A6. *Virology* 2002; 301:374–380

8 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; 37:2483–2487

© 2007 The Author(s)

9 Hokynar K, Soderlund-Venermo M, Pesonen M, Ranki A, Kiviluoto O, Partio EK, Hedman K: A new parvovirus genotype persistent in human skin. *Virology* 2002; 302:224–228
10 Servant A, Laperche S, Lallemand F, Marinho V, De Saint MG, Meritet JF, Garbarg-Chenon A: Genetic diversity within human erythroviruses: identification of three genotypes. *J Virol* 2002; 76:9124–9134
11 Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds): *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses (ICTV)*. London, UK, Elsevier, 2005:360–361
12 Nubling M, Buchheit K-H: Results from recent EDQM/OMCL PTS studies (PTS052 and PTS064) on B19 DNA NAT testing of plasma pools; Presentation at the SoGAT meeting, 14 June 2006, Bern, Switzerland
13 Cohen BJ, Gandhi J, Clewley JP: Genetic variants of parvovirus B19 identified in the United Kingdom: implications for diagnostic testing. *J Clin Virol* 2006; 36:152–155
14 Hokynar K, Norja P, Laitinen H, Palomaki P, Garbarg-Chenon A, Ranki A, Hedman K, Soderlund-Venermo M: Detection and differentiation of human parvovirus variants by commercial quantitative real-time PCR tests. *J Clin Microbiol* 2004; 42:2013–2019
15 Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, van der Noordaa J: Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* 1990; 28:495–503
16 Johnson MP, Haupt LM, Griffiths LR: Locked nucleic acid (LNA) single nucleotide polymorphism (SNP) genotype analysis and validation using real-time PCR. *Nucleic Acids Res* 2004; 32:e55
17 Latorra D, Arar K, Hurley JM: Design considerations and effects of LNA in PCR primers. *Mol Cell Probes* 2003; 17:253–259
18 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; 79:14197–14206
19 Heegaard ED, Panum JI, Christensen J: Novel PCR assay for differential detection and screening of erythrovirus B19 and erythrovirus V9. *J Med Virol* 2001; 65:362–367
20 Candotti D, Etiz N, Parsyan A, Allain JP: Identification and characterization of persistent human erythrovirus infection in blood donor samples. *J Virol* 2004; 78:12169–12178
21 Gierman TM: Industry experience with B19 virus reagents, test kits, and in-process testing of plasma donor samples. EDQM B19 virus test kit meeting, 9 November 2006, Strasbourg
22 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; 92:838–845
23 Norja P, Hokynar K, Aaltonen LM, Chen R, Ranki A, Partio EK, Kiviluoto O, Davidkin I, Leivo T, Eis-Hubinger AM, Schneider B, Fischer HP, Tolba R, Vapalahti O, Vaheri A, Soderlund-Venermo M, Hedman K: Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. *Proc Natl Acad Sci USA* 2006; 103:7450–7453
24 Baylis SA, Fryer JF, Grabarczyk P: Effects of probe binding mutations in an assay designed to detect parvovirus B19: implications for the quantitation of different virus genotypes. *J Virol Methods* 2007; 139:97–99
25 Teupser D, Rupprecht W, Lohse P, Thiery J: Fluorescence-based detection of the CETP TaqIB polymorphism: false positives with the TaqMan-based exonuclease assay attributable to a previously unknown gene variant. *Clin Chem* 2001; 47:852–857
26 Shackelton LA, Holmes EC: Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. *J Virol* 2006; 80:3666–3669

© 2007 The Author(s)

別紙様式第 2-1

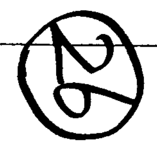
医薬品
医薬部外品　研究報告　調査報告書
化粧品

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。



BYL-2008-029
080123
人血清アルブミン

Vox Sanguinis (2007) 93, 216–222

ORIGINAL PAPER

© 2007 Biotrin International
Journal compilation © 2007 Blackwell Publishing Ltd.
DOI: 10.1111/j.1423-0410.2007.00956.x

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.

Received: 19 February 2007, revised 14 June 2007, accepted 16 June 2007, published online 7 August 2007

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

Introduction

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

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].

© 2007 Biotrin International

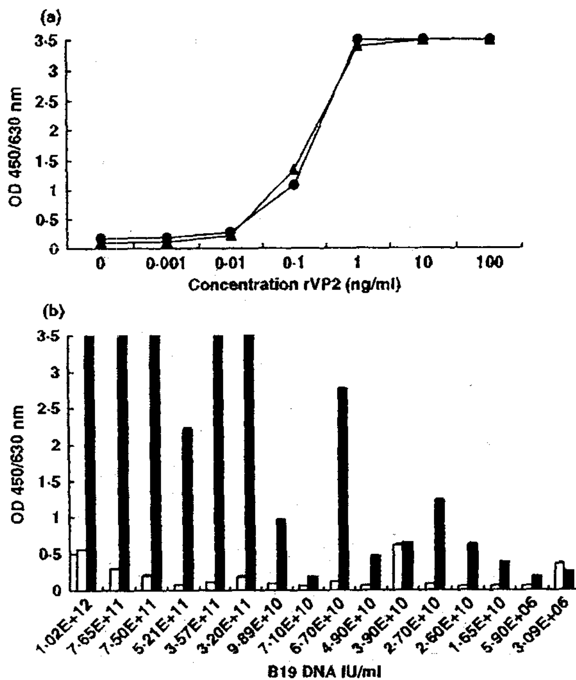


Fig. 1 Determination of B19 antigen enzyme immunoassay (EIA) assay sensitivity. (a) Two independent batches of recombinant capsid VP2 (rVP2), V056 (circles) and V057 (triangles) were decimally diluted to determine assay sensitivity. (b) Comparison of specimen diluents used in the detection of B19 viral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

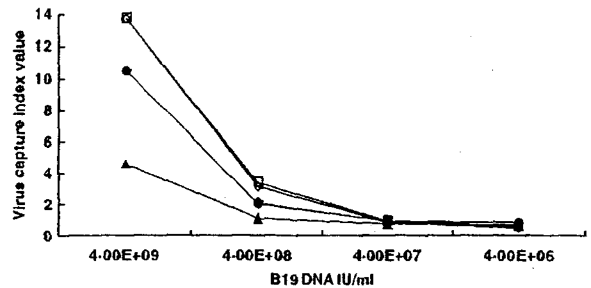


Fig. 2 Determination of antigen assay sensitivity using titrations of polymerase chain reaction (PCR)-quantified viraemic specimens. Viraemic plasma Bt72 (diamonds), Bt73 (squares), Bt80 (triangles) and genotype 2 Bt81 (circles) were decimally diluted in B19 negative serum to determine assay cut-off.

Results

Assay optimization and validation

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

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

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

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

© 2007 Biotrin International

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

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

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

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

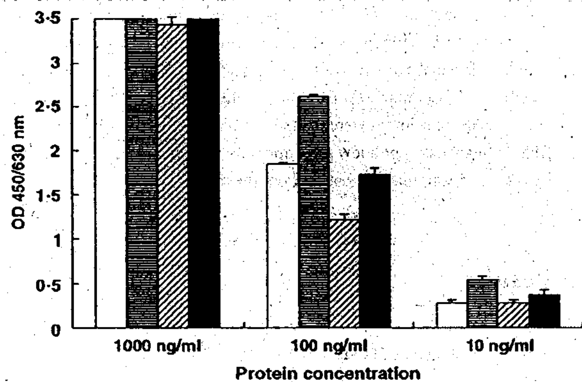


Fig. 3 Comparison of erythrovirus genotype 1 and 3 VP2 reactivity in the antigen enzyme immunoassay (EIA). Genotype 1 (clear and horizontal lined bars) and genotype 3 (diagonal lined and filled bars) recombinant VP2 was decimally diluted in either Tris-buffered saline Tween-20 (TBST) (clear and diagonal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

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

Donor sample evaluation

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

© 2007 Biotrin International

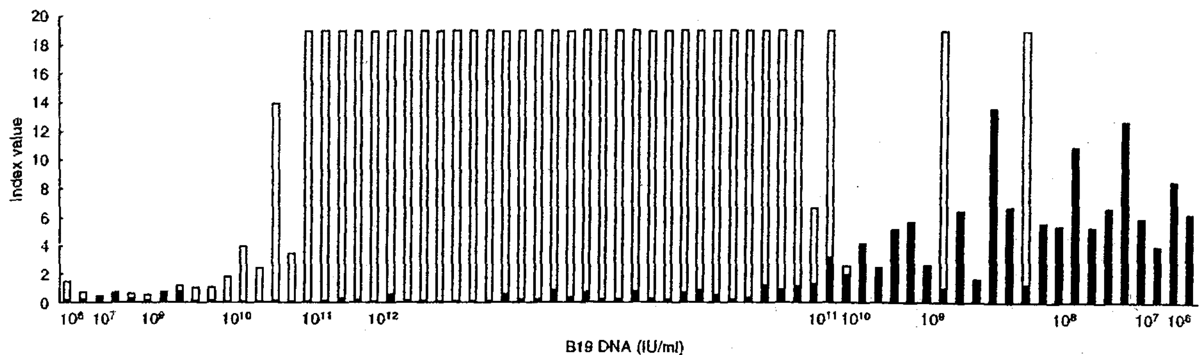


Fig. 4 A summary of the B19 antigen enzyme immunoassay (EIA) and immunoglobulin M (IgM) EIA reactivity of the panel of viraemic donors. An index value (IV) > 1·1 (denoted by line) is considered positive on both the B19 IgM EIA (filled bars) and antigen EIA (clear bars). The *y*-axis was truncated for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

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

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

Discussion

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

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

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

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

© 2007 Biotrin International

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

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

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

Acknowledgements

We are grateful to Jean Pierre Allain (University of Cambridge, UK) for providing recombinant genotype 3 VP2 protein. Financial support: Quality of Life and Management of Living Resources Program, Commission of the European Communities (project QLK2-CT-2001-00877 'Human Parvovirus Infection: Towards Improved Understanding, Diagnosis and Therapy').

References

1 Kurtzman GJ, Cohen BJ, Meyers P, Amunullah A, Young NS: Persistent B19 parvovirus infection as a cause of severe chronic anemia in children with acute lymphocytic leukaemia. *Lancet* 1988; 2:1159-1162

2 Wildig J, Michon P, Siba P, Mellombo M, Ura A, Mueller I, Cossart Y: Parvovirus B19 infection contributes to severe anemia in young children in Papua New Guinea. *J Infect Dis* 2006; 194:146-153

3 Enders M, Weidner A, Zoellner I, Searle K, Enders G: Fetal morbidity and mortality after acute human parvovirus B19 infection in pregnancy: prospective evaluation of 1018 cases. *Prenat Diagn* 2004; 24:513-518

4 European Pharmacopoeia: *European Pharmacopoeia Monograph of Human anti-D Immunoglobulin*. European Pharmacopoeia, Strasbourg, France, 2004; Document 01:0557

5 Groeneveld K, van der Noordaa J: Blood products and parvovirus B19. *Neth J Med* 2003; 61:154-156

6 Prowse C, Ludlam CA, Yap PL: Human parvovirus B19 and blood products. *Vox Sang* 1997; 72:1-10

7 Santagostino E, Mannucci PM, Gringeri A, Azzi A, Morfini M, Musso R, Santoro R, Schiavoni M: Transmission of parvovirus B19 by coagulation factor concentrates exposed to 100 degrees C heat after lyophilization. *Transfusion* 1997; 37:517-522

8 Laub R, Stengers P: Parvovirus B19 and blood products. *Pathol Biol* 2002; 50:339-348

9 Hausler M, Scheithauer S, Ritter K, Kleines M: Molecular diagnosis of Epstein-Barr virus. *Expert Rev Mol Diagn* 2003; 3:81-92

10 Baylis SA, Shah N, Minor PD: Evaluation of different assays for the detection of parvovirus B19 DNA in human plasma. *J Virol Methods* 2004; 121:7-16

11 Sakata H, Ihara H, Sato S, Kato T, Ikeda H, Sekiguchi S: Efficiency of donor screening for human parvovirus B19 by the receptor-mediated hemagglutination assay method. *Vox Sang* 1999; 77:197-203

12 Koppelman M, Cuypers T, Emrich T, Zaaijer H: Quantitative real-time detection of parvovirus B19 DNA in plasma. *Transfusion* 2004; 44:97-103

13 Aubin JT, Defer C, Vidaud M, Maniez Montreuil M, Flan B: Large-scale screening for human parvovirus B19 DNA by PCR: application to the quality control of plasma for fractionation. *Vox Sang* 2000; 78:7-12

14 Wong S, Brown KE: Development of an improved method of detection of infectious parvovirus B19. *J Clin Virol* 2006; 35:407-413

15 Takeda Y, Wakisaka A, Noguchi K, Murozuka T, Katsubayashi Y, Matsumoto S, Tomono T, Nishioka K: Receptor-mediated haemagglutination screening and reduction in the viral load of parvovirus B19 DNA in immunopurified Factor VIII concentrate (Cross Eight M). *Vox Sang* 2001; 81:266-268

16 Doyle S, Corcoran A: The immune response to parvovirus B19 exposure in previously seronegative and seropositive individuals. *J Infect Dis* 2006; 194:154-158

17 O'Keeffe S, O'Leary D, Doyle S, Kilty C, Kerr S: The detection of parvovirus B19 in human sera using antigen-capture EIA. Poster presented at the Society for General Microbiology (Irish Branch), National University of Ireland, Maynooth, Co. Kildare, Ireland, 24-25 April 2003

18 Lowin T, Raab U, Schroeder J, Franssila R, Modrow S: Parvovirus B19 VP2-proteins produced in *Saccharomyces cerevisiae*: comparison with VP2-particles produced by baculovirus-derived vectors. *J Vet Med B Infect Dis Vet Public Health* 2005; 52:348-352

19 Doyle S, Kerr S, O'Keeffe G, O'Carroll D, Daly P, Kilty C: Detection of parvovirus B19 IgM by antibody capture enzyme immunoassay: receiver operating characteristic analysis. *J Virol Methods* 2000; 90:143-152

© 2007 Biotrin International

20 Beersma MF, Claas EC, Sopaheluakan T, Kroes AC: Parvovirus B19 viral loads in relation to VP1 and VP2 antibody responses in diagnostic blood samples. *J Clin Virol* 2005; 34:71–75

21 Kerr S, O'Keeffe G, Kilty C, Doyle S: Undenatured parvovirus B19 antigens are essential for the accurate detection of parvovirus IgG. *J Med Virol* 1999; 57:179–185

22 Hermanson GT: Heterobifunctional Cross-Linkers; in *Bioconjugate Techniques*. San Diego, Academic Press, 1996:228–286

23 Saldanha J, Lelie N, Yu MW, Heath A: B19 Collaborative Study Group: Establishment of the first World Health Organization International Standard for human parvovirus B19 DNA nucleic acid amplification techniques. *Vox Sang* 2002; 82:24–31

24 Boschetti N, Niederhauser I, Kempf C, Stuhler A, Lower J, Blumel J: Different susceptibility of B19 virus and mice minute virus to low pH treatment. *Transfusion* 2004; 44:1079–1086

25 Nguyen QT, Wong S, Heegaard ED, Brown K: Identification and characterisation of a second novel human erythrovirus variant, A6. *Virology* 2002; 301:374–380

26 Servant A, Laperche S, Lallemand F, Marinho V, De Saint Maur G, Meritet JF, Garbarg-Chenon A: Genetic diversity within human erythroviruses: identification of three genotypes. *J Virol* 2002; 76:9124–9134

27 Hokynar K, Ekman A, Kantola K, Kakkola L, Hedman L, Bonden H, Aberham C, Gessner M, Garbarg-Chenon A, Hedman K, Soderlund-Venermo M: Biological and immunological relation of the three variants of human parvovirus B19. Poster presented at the XIth Parvovirus Conference, Les Diablerets, Switzerland, 27–31 August 2006

28 Cassinotti P, Siegl G: Quantitative evidence for persistence of human parvovirus B19 DNA in an immunocompetent individual. *Eur J Clin Microbiol Infect Dis* 2000; 19:886–887

29 Anderson MJ, Higgins PG, Davis LR, Willman JS, Jones SE, Kidd IM, Pattison JR, Tyrrell DA: Experimental parvovirus infection in humans. *J Infect Dis* 1985; 152:257–265

© 2007 Biotrin International

別紙様式第 2-1

別紙 3-6

医薬品
医薬部外品　研究報告　調査報告書
化粧品

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2007 年 12 月 5 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	Standardization of nucleic acid amplification technique (NAT)-based assays for different genotypes of parvovirus B19: a meeting summary. Baylis, S.A. Vox Sanguinis, 94, 74-80 (2008)	公表国 英国	
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
ヒト血液製剤のパルボウイルス B19 汚染に関するゲノム増幅法の標準化についての国際ワーキンググループ会議（2007 年 3 月開催）で協議された議題の要約である。 パルボウイルス B19 の新規の遺伝子型が発見されていることから，規制基準を満たすためには核酸増幅法（NAT）による血漿プールのスクリーニング手順を更新する必要がある。そのため，本会議は，管理分析機関及び血漿分画製剤の製造者間でのパルボウイルス B19 の種々の遺伝子型の検出及び定量結果を統一する方法を見いだす目的で開催された。パルボウイルス B19 の全 3 種の遺伝子型は極めて似通っており，in vitro 試験で感染性の差は認められなかった。遺伝子型 1 及び 2 は，熱又は低 pH 条件に対し同等に不活化されることが知られている。さらに，先に示した文献［BYL-2008-0297］に記載の結果も本会議で提示された；米国人及びヨーロッパ人の血漿ドナーにおける遺伝子型 2 及び 3 の保有率は非常に低く，ガーナにおけるパルボウイルス B19 感染は大部分が遺伝子型 3 に起因していた。 本会議では，特性が十分に明らかになっている標準物質を用いたアッセイの標準化について合意が得られた。これにより，パルボウイルス B19 の種々の遺伝子型を示す血漿検体パネルが作成されることが示唆される。また，会議中，パルボウイルス B19 株の新規 DNA 配列がある場合はデータベースに蓄積し，閲覧可能な状態にしておくべきであることも強調されていた。	BYL-2008-0304

報告企業の意見	今後の対応
パルボウイルス B19 の新規の遺伝子型が発見されており，規制基準に対応するためには，パルボウイルス B19 の検出アッセイを新たに開発するか，又は高感度，或いは特異的なアッセイにする必要があると考えられる。 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。	引き続き，パルボウイルス B19 の亜型の検出や疫学に関する研究の進展の情報収集に努める。

BYL-2008-
08012
人血清アル

Vox Sanguinis (2008) 94, 74–80

© 2007 The Author(s)
Journal compilation © 2007 Blackwell Publishing Ltd.
DOI: 10.1111/j.1423-0410.2007.00992.x

REPORT

Standardization of nucleic acid amplification technique (NAT)-based assays for different genotypes of parvovirus B19: a meeting summary

S. A. Baylis
Division of Virology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, UK

Vox Sanguinis

An extraordinary meeting of the International Working Group on the Standardization of Genome Amplification Techniques for the safety testing of blood, tissues and organs for blood borne pathogens was held on 2 March 2007, at the National Institute for Biological Standards and Control. The aim of the meeting was to investigate ways to harmonize results obtained for the detection and quantification of different genotypes of parvovirus B19 (B19V) DNA by control laboratories and manufacturers of plasma derivatives. The meeting explored issues of B19V such as the classification of B19V strains, the prevalence and distribution of different genotypes, the clinical and biological significance of different genotypes, the detection of different genotypes in plasma-derived products, and their susceptibility to virus-inactivation procedures. At this meeting and through subsequent studies, high titre, high volume samples have been identified representing different genotypes of B19V, which will be evaluated by collaborative study to prepare reference panels for the purposes of assay validation.

Received: 6 July 2007, revised 20 August 2007, accepted 22 August 2007, published online 24 September 2007

Key words: erythrovirus B19V, plasma screening, B19V variants.

Introduction

Since 2004, European regulatory requirements have meant that plasma used in the production of anti-D immunoglobulin and pooled human plasma treated for virus inactivation must be screened to ensure that levels of parvovirus B19 (B19V) DNA do not exceed 10 IU/μl [1–3]. Plasma donations containing high titres of B19V are removed by the manufacturers of plasma derivatives, and the appropriate pools are tested by a group of European Official Medicines Control Laboratories (OMCLs) for subsequent batch release. Screening is performed using nucleic acid amplification technique (NAT)-based assays for B19V DNA. The introduction of these regulatory requirements was underpinned by the establishment of the first World Health Organization (WHO) International Standard for B19V DNA (NIBSC code 99/800) [4]. The discovery that B19V was more genetically diverse than was originally thought, forming three genotypes [5] has led to a review of testing procedures. Strains, representing each of the two more recently identified genotypes, have now been formally classified as B19V by the International Committee on the Taxonomy of Viruses (ICTV) [6]. This classification has led to regulatory issues. The guidelines for validation of quantitiative NAT assays for B19V, due to be published in the European Pharmacopoeia (Ph. Eur.), recommend that all genotypes of B19V should be detected. Recent Proficiency Testing Schemes (PTS), run by the European Directorate for the Quality of Medicines (EDQM), who coordinate the OMCL network, have highlighted discrepant results, when samples representing different genotypes of B19V have been included in the panels [7]. This was discussed further at a meeting held at the EDQM in Strasbourg on 9 November 2006, which focused on some of the issues with the types of commercial NAT assays available for the detection and quantification of B19V DNA. In an effort to harmonize results obtained by control laboratories and plasma fractionators, an extraordinary meeting of Standardization of Genome Amplification Techniques (SoGAT) was held at National Institute for Biological Standards and Control (NIBSC) on 2 March 2007. The aim of the meeting was to identify ways to provide appropriate reference materials, to support the

Correspondence: Sally A. Baylis, Division of Virology, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK
E-mail: sbaylis@nibsc.ac.uk

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

© 2007 The Author(s)

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 VIIIs, 81% were contaminated with genotype 1 B19V, and 14% were contaminated with genotype 2. In the more recent factor VIIIs, 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 ~10^7 and ~10^5 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 10^3 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 (10^6) 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

© 2007 The Author(s)

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 (Ct) 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 ~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]

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 > 2×10^5 IU/ml and genotypes 2 and 3 titres > 5×10^6 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 (~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

[1] Dr 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, NA, 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.

© 2007 The Author(s)

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 ~7 $\log_{10}$ IU/m. 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 ~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 know 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.

References

1 Anonymous: Council of Europe: Human anti-D immunoglobulin, monograph 0557; in *European Pharmacopoeia*, 6th edn. Strasbourg, France, Council of Europe, 2006

2 Anonymous: Council of Europe Human anti-D immunoglobulin for intravenous administration, monograph 1527; in *European Pharmacopoeia*, 6th edn. Strasbourg, France, Council of Europe, 2006

© 2007 The Author(s)
Journal compilation © 2007 Blackwell Publishing Ltd.,

3 Anonymous: Council of Europe Human plasma (pooled and treated for virus inactivation), monograph 1646; in *European Pharmacopoeia*, 6th edn. Strasbourg, France, Council of Europe, 2006

4 Saldanha J, Lelie N, Yu MW, Heath A; and the B19 Collaborative Study Group: Establishment of the first World Health Organization International Standard for human parvovirus B19 DNA nucleic acid amplification techniques. *Vox Sang* 2002; 82:24–31

5 Servant A, Laperche S, Lallemand F, Marinho V, De Saint Maur G, Meritet JF, Garbarg-Chenon A: Genetic diversity within human erythroviruses: identification of three genotypes. *J Virol* 2002; 76:9124–9134

6 Tattersall P, Bergoin M, Bloom ME, Brown KE, Linden RM, Muzyczka N, Parrish CR, Tijssen P: Parvoviridae; in Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds): *Virus Taxonomy: Eighth report of the International Committee on Taxonomy of Viruses*. New York, Academic Press, 2005;353–369

7 Nübling M, Buchheit K-H: Results from recent EDQM/OMCL PTS studies (PTS052 and PTS064) on B19 DNA NAT testing of plasma pools; Presentation at the XIX SoGAT meeting, 14 June 2006, Bern, Switzerland

8 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; 37:2483–2487

9 Nguyen QT, Wong S, Heegaard ED, Brown KE: Identification and characterization of a second novel human erythrovirus variant, A6. *Virology* 2002; 301:374–380

10 Hokynar K, Söderlund-Venermo M, Pesonen M, Ranki A, Kiviluoto O, Partio EK, Hedman K: A new parvovirus genotype persistent in human skin. *Virology* 2002; 302:224–228

11 Jones MS, Kapoor A, Lukashov VV, Simmonds P, Hecht F, Delwart E: New DNA viruses identified in patients with acute viral infection syndrome. *J Virol* 2005; 79:8230–8236

12 Fryer JF, Delwart E, Bernardin F, Tuke PW, Lukashov VV, Baylis SA: Analysis of two human parvovirus PARV4 genotypes identified in human plasma for fractionation. *J Gen Virol* 2007; 88:2162–2167

13 Allander T, Tammi M, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B: Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci USA* 2005; 102:12891–12896

14 PA/PH/OMCL (03) 38 DEF: OMCL Guideline for validation of nucleic acid amplification techniques (NAT) for quantitiation of B19 virus DNA in plasma pools; in *Biological Substances Submitted to the Official Control Authority Batch Release*. Strasbourg, France, Council of Europe; 2006

15 Wu C, Mason B, Jong J, Erdman D, McKernan L, Oakley M, Soucie M, Evatt B, Yu MW: Parvovirus B19 transmission by a high-purity factor VIII concentrate. *Transfusion* 2005; 45:1003–1010

16 Norja P, Hokynar K, Aaltonen LM, Chen R, Ranki A, Partio EK, Kiviluoto O, Davidkin I, Leivo T, Eis-Hübinger AM, Schneider B, Fischer HP, Tolba R, Vapalahti O, Vaheri A, Söderlund-Venermo M, Hedman K: Bioportfolio: lifelong persistence of variant and prototypic erythrovirus DNA genomes in human tissue. *Proc Natl Acad Sci USA* 2006; 103:7450–7453

17 Ekman A, Hokynar K, Kakkola L, Kantola K, Hedman L, Bondén H, Gessner M, Aberham C, Norja P, Miettinen S, Hedman K, Söderlund-Venermo M: Biological and immunological relations among human parvovirus B19 genotypes 1 to 3. *J Virol* 2007; 81:6927–6935

18 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 to 3. *J Med Virol* 2005; 75:161–169

19 Candotti D, Etiz N, Parsyan A, Allain JP: Identification and characterization of persistent human erythrovirus infection in blood donor samples. *J Virol* 2004; 78:12169–12178

20 Parsyan A, Szmaragd C, Allain JP, Candotti D: Identification and genetic diversity of two human parvovirus B19 genotype 3 subtypes. *J Gen Virol* 2007; 88:428–431

21 Shackelton LA, Parrish CR, Truyen U, Holmes EC: High rate of viral evolution associated with the emergence of carnivore parvovirus. *Proc Natl Acad Sci USA* 2005; 102:379–384

22 Shackelton LA, Holmes EC: Phylogenetic evidence for the rapid evolution of human B19 erythrovirus. *J Virol* 2006; 80:3666–3669

23 Schneider B, Becker M, Brackmann HH, Eis-Hübinger AM: Contamination of coagulation factor concentrates with human parvovirus B19 genotype 1 and 2. *Thromb Haemost* 2004; 92:838–845

24 Geng Y, Wu C, Bhattacharyya SP, Tan D, Guo Z, Yu MW: Parvovirus B19 DNA in factor VIII concentrates: effects of manufacturing procedures and B19 NAT screening. *Transfusion* 2007; 47:883–889

25 Blümel J, Eis-Hübinger AM, Stühler A, Bönsch C, Gessner M, Löwer J: Characterization of parvovirus B19 genotype 2 in KU812Ep6 cells. *J Virol* 2005; 79:14197–14206

26 Boschetti N, Niederhauser I, Kempf C, Stühler A, Löwer J, Blümel J: Different susceptibility of B19 virus and mice minute virus to low pH treatment. *Transfusion* 2004; 44:1079–1086

27 Allain JP, Hsu J, Pranmeth M, Hanson D, Stassinopoulos A, Fischetti L, Corash L, Lin L: Quantification of viral inactivation by photochemical treatment with amotosalen and UV A light, using a novel polymerase chain reaction inhibition method with preamplification. *J Infect Dis* 2006; 194:1737–1744

28 Baylis SA, Shah N, Minor PD: Evaluation of different assays for the detection of parvovirus B19 DNA in human plasma. *J Virol Methods* 2004; 121:7–16

29 Hokynar K, Norja P, Laitinen H, Palomäki P, Garbarg-Chenon A, Ranki A, Hedman K, Söderlund-Venermo M: Detection and differentiation of human parvovirus variants by commercial quantitative real-time PCR tests. *J Clin Microbiol* 2004; 42:2013–2019

30 Cohen BJ, Gandhi J, Clewley JP: Genetic variants of parvovirus B19 identified in the United Kingdom: implications for diagnostic testing. *J Clin Virol* 2006; 36:152–155

31 Koppelman MHGM, Rood IGH, Fryer JF, Baylis SA, Cuypers HTM: Parvovirus B19 genotype 1 and 2 detection with real-time PCR assays. *Vox Sang* 2007; 93:208–215

32 Sanabani S, Neto WK, Pereira J, Sabino EC: Sequence variability of human erythroviruses present in bone marrow of Brazilian patients with various parvovirus B19-related hematological symptoms. *J Clin Microbiol* 2006; 44:604–606

© 2007 The Author(s)
Journal compilation © 2007 Blackwell Publishing Ltd.,

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).

© 2007 The Author(s)
Journal compilation © 2007 Blackwell Publishing Ltd.,

医薬品
医薬部外品　研究報告　調査報告書
化粧品

識別番号・報告回数			報告日	第一報入手日 2008年2月26日	新医薬品等の区分 該当なし	厚生労働省処理欄
一般的名称	①乾燥抗HBs人免疫グロブリン ②ポリエチレングリコール処理抗HBs人免疫グロブリン		研究報告の公表状況	Science 2008; 319: 1096-1100	公表国 アメリカ	
販売名（企業名）	①ヘブスブリン（ベネシス） ②静注用ヘブスブリン－IH（ベネシス）					

研究報告の概要

メルケル細胞癌（Merkel cell carcinoma: MCC）は、稀ではあるが進行の早いヒトの皮膚がんであり、主に高齢者や免疫抑制者にみられる。われわれは今回、digital transcriptome subtraction（DTS）法を用いてMCC検体について調べ、新種のポリオーマウイルスを同定し、メルケル細胞ポリオーマウイルス（MCVまたはMCPyV）と命名した。このウイルスは、MCC腫瘍の10検体のうち8検体（80%）で検出されたが、体内のさまざまな部位から採取した対照組織では59検体中5検体（8%）、対照皮膚組織では25検体中4検体（16%）でしか検出されなかった。MCV陽性であった8MCC検体のうち6検体では、ウイルスDNAは腫瘍ゲノム内に組み込まれており、そのパターンより、MCVの感染と組み込みにより腫瘍細胞のクローン増殖となったことが示唆された。したがって、MCVはMCCの発生に寄与する因子であると考えられる。

使用上の注意記載状況・その他参考事項等

代表として静注用ヘブスブリン－IHの記載を示す。
2. 重要な基本的注意
(1)本剤の原材料となる血液については、HBs抗原、抗HCV抗体、抗HIV-1抗体、抗HIV-2抗体陰性で、かつALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV及びHCVについて核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該NATの検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した高力価の抗HBs抗体を含有する血漿を原料として、Cohnの低温エタノール分画で得た画分からポリエチレングリコール4000処理、DEAEセファデックス処理等により抗HBs人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において60℃、10時間の液状加熱処理及びろ過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見

メルケル細胞癌の発生に寄与すると考えられる新規のポリオーマウイルスを同定したとの報告である。
ポリオーマウイルスは、直径40nmのエンベロープを有しないDNAウイルスである。
静注用ヘブスブリン-IHについては、万一本剤の原料血漿にポリオーマウイルスが混入したとしても、EMC及びCPVをモデルウイルスとしたウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。
ヘブスブリンについては、EMC及びCPVをモデルウイルスとしたウイルスバリデーション試験成績からは、製造工程において不活化・除去が十分であるとは説明困難であるため、ポリオーマウイルスの原血漿への混入が判明した場合にはその事実を総合機構及び厚生労働省に報告する。

今後の対応

ポリオーマウイルスに関連する情報については、今後も注視することとする。

mains of TRF1 and TRF2, these structural variations emphasize that the TRFH domain is a versatile framework for interactions with different proteins.

The crystal structure of the $TRF2_{TRFH}$-$Apollo_{TBM}$ complex is corroborated by mutagenesis. Mutations of the conserved hydrophobic residues of Apollo (F504, L506, and P508) or TRF2 (*F120*) completely abolished the interaction both in vitro and in vivo (Fig. 4, F and G). We further assayed the cellular localization of wild-type and mutant Apollo by expressing hemagglutinin (HA)–tagged proteins in human telomerase reverse transcriptase (hTERT)–immortalized human BJ fibroblasts. Although wild-type Apollo showed the expected telomere localization, the L506E/P508A double mutant was distributed throughout the nucleoplasm with no obvious accumulation at telomeres (Fig. 4H). This result confirms the structural information and indicates that the binding of Apollo to the TRFH domain of TRF2 is required for the telomeric localization of Apollo.

We next asked whether other shelterin-associated proteins might contain the F/Y-X-L-X-P motif suggestive of an interaction with the TRFH domain of TRF1 or TRF2. We identified this motif in PinX1, originally identified as a TRF1-interacting protein in a yeast two-hybrid screen (*6*). An 11-residue fragment of PinX1 (R287-D-F-T-L-K-P-K-K-R-R297), referred to as $PinX1_{TBM}$, closely resembles $TIN2_{TBM}$ (fig. S12A), suggesting that it may bind to $TRF1_{TRFH}$ in the same fashion as does $TIN2_{TBM}$. ITC data confirmed the $TRF1_{TRFH}$-$PinX1_{TBM}$ interaction, whereas no measurable interaction was observed between $TRF2_{TRFH}$ and $PinX1_{TBM}$ (fig. S12B). Mutagenesis studies showed that PinX1-L291 and TRF1-*F142* are critical for the interaction, whereas PinX1-P293 is not (fig. S12C). These results are consistent with those of the $TRF1_{TRFH}$-$TIN2_{TBM}$ interaction (Fig. 2D) and indicate that PinX1, like TIN2, binds the TRFH domain of TRF1 but not TRF2. Protein sequence database searches showed many instances of telomere-associated proteins containing the F/Y-X-L-X-P motif (fig. S13). Future studies are needed to address whether this motif mediates the TRF1/TRF2 binding of these telomere-associated proteins in vivo.

Our results indicate that binding to the TRFH docking site involves the sequence F/Y-X-L-X-P in shelterin-associated proteins, which contacts the same molecular recognition surface of the TRFH domains of TRF1 and TRF2 with distinct specificities. Because TRF1 and TRF2 play different roles in telomere length homeostasis and telomere protection (*1*), we propose that the TRFH domains of TRF1 and TRF2 function as telomeric protein docking sites that recruit different shelterin-associated factors with distinct functions to the chromosome ends.

References and Notes

1. T. de Lange, *Genes Dev.* **19**, 2100 (2005).
2. M. van Overbeek, T. de Lange, *Curr. Biol.* **16**, 1295 (2006).
3. X. D. Zhu, B. Kuster, M. Mann, J. H. Petrini, T. de Lange, *Nat. Genet.* **25**, 347 (2000).
4. X. D. Zhu *et al.*, *Mol. Cell* **12**, 1489 (2003).
5. S. Smith, I. Giriat, A. Schmitt, T. de Lange, *Science* **282**, 1484 (1998).
6. X. Z. Zhou, K. P. Lu, *Cell* **107**, 347 (2001).
7. T. Nishikawa *et al.*, *Structure* **9**, 1237 (2001).
8. R. Court, L. Chapman, L. Fairall, D. Rhodes, *EMBO Rep.* **6**, 39 (2005).
9. B. Li, S. Oestreich, T. de Lange, *Cell* **101**, 471 (2000).
10. A. Bianchi, S. Smith, L. Chong, P. Elias, T. de Lange, *EMBO J.* **16**, 1785 (1997).
11. L. Fairall, L. Chapman, H. Moss, T. de Lange, D. Rhodes, *Mol. Cell* **8**, 351 (2001).
12. S. H. Kim, P. Kaminker, J. Campisi, *Nat. Genet.* **23**, 405 (1999).
13. C. Lenain *et al.*, *Curr. Biol.* **16**, 1303 (2006).
14. T. H. Lee, K. Perrem, J. W. Harper, K. P. Lu, X. Z. Zhou, *J. Biol. Chem.* **281**, 759 (2006).
15. S. H. Kim *et al.*, *J. Biol. Chem.* **279**, 43799 (2004).
16. J. Z. Ye *et al.*, *J. Biol. Chem.* **279**, 47264 (2004).
17. Materials and methods are available as supporting material on *Science* Online.
18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. P. Fotiadou, O. Henegariu, J. B. Sweasy, *Cancer Res.* **64**, 3830 (2004).

20. Coordinates and structure factor amplitudes have been deposited in the Protein Data Bank with access numbers 3BQO ($TRF1_{TRFH}$-$TIN2_{TBM}$), 3BU8 ($TRF2_{TRFH}$-$TIN2_{TBM}$), and 3BUA ($TRF2_{TRFH}$-$Apollo_{TBM}$). We thank F. Wang and K. Wan for assistance. Work was supported by an NIH grant (to T.de L.) and an American Cancer Society Research Scholar grant and a Sidney Kimmel Scholar award (to M.L.). Use of Life Sciences Collaborative Access Team Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (grant 085P1000817). Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under contract no. DE-AC02-06CH11357.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1151804/DC1
Materials and Methods
SOM Text
Figs. S1 to S14
Table S1
References

16 October 2007; accepted 7 January 2008
Published online 17 January 2008;
10.1126/science.1151804
Include this information when citing this paper.

Clonal Integration of a Polyomavirus in Human Merkel Cell Carcinoma

Huichen Feng, Masahiro Shuda, Yuan Chang,* Patrick S. Moore*

Merkel cell carcinoma (MCC) is a rare but aggressive human skin cancer that typically affects elderly and immunosuppressed individuals, a feature suggestive of an infectious origin. We studied MCC samples by digital transcriptome subtraction and detected a fusion transcript between a previously undescribed virus T antigen and a human receptor tyrosine phosphatase. Further investigation led to identification and sequence analysis of the 5387–base-pair genome of a previously unknown polyomavirus that we call Merkel cell polyomavirus (MCV or MCPyV). MCV sequences were detected in 8 of 10 (80%) MCC tumors but only 5 of 59 (8%) control tissues from various body sites and 4 of 25 (16%) control skin tissues. In six of eight MCV-positive MCCs, viral DNA was integrated within the tumor genome in a clonal pattern, suggesting that MCV infection and integration preceded clonal expansion of the tumor cells. Thus, MCV may be a contributing factor in the pathogenesis of MCC.

Polyomaviruses have been suspected as potential etiologic agents in human cancer since the discovery of murine polyoma virus (MuPyV) by Gross in 1953 (*1*). However, although polyomavirus infections can produce tumors in animal models, there is no conclusive evidence that they play a role in human cancers (*2*). These small double-stranded DNA viruses [~5200 base pairs (bp)] encode a variably spliced oncoprotein, the tumor (T) antigen (*3*, *4*), and are divided into three genetically distinct groups: (i) avian polyomaviruses, (ii) mammalian viruses related to MuPyV, and (iii) mammalian polyomaviruses related to simian virus 40 (SV40) (*5*). All four known human polyomaviruses [BK virus (BKV), JCV, KIV, and WUV (*6*, *7*)] belong to the SV40 subgroup. In animals, integration of polyomavirus DNA into the host genome often precedes tumor formation (*8*).

Merkel cell carcinoma (MCC) is a neuroectodermal tumor arising from mechanoreceptor Merkel cells (Fig. 1A). MCC is rare, but its incidence has tripled over the past 2 decades in the United States to 1500 cases per year (*9*). It is one of the most aggressive forms of skin cancer; about 50% of advanced MCC patients

Molecular Virology Program, University of Pittsburgh Cancer Institute, University of Pittsburgh, 5117 Centre Avenue, Suite 1.8, Pittsburgh, PA 15213, USA.

*These authors contributed equally to this work. To whom correspondence should be addressed. E-mail: yc70@pitt.edu (Y.C.); psm9@pitt.edu (P.S.M.)

live 9 months or less. Gene expression profiling studies indicate that MCC may comprise two or more clinically similar diseases with distinct etiologies (10). Like Kaposi's sarcoma (KS), MCC occurs more frequently than expected among immunosuppressed transplant and AIDS patients (11). These similarities to KS, an immune-related tumor caused by KS-associated herpesvirus (12), raise the possibility that MCC may also have an infectious origin.

To search for viral sequences in MCC, we used digital transcriptome subtraction (DTS), a methodology we developed that can identify foreign transcripts by using human high-throughput cDNA sequencing data (13). We generated two cDNA libraries from a total of four anonymized MCC tumors. One library was prepared with the use of mRNA from a single tumor (MCC347), and the other was prepared with mRNA pooled from three tumors (MCC337, 343, and 346) to increase the likelihood of detecting rare viral sequences (table S1).

From these two libraries, we respectively pyrosequenced 216,599 and 179,135 cDNA sequences (~150 to 200 bp). These 395,734 cDNA sequences were trimmed with LUCY stringency equivalent to PHRED scores of 20 or higher (14). Copolymers of adenine or thymidine [poly(A) and poly (T), respectively], dust (low-complexity), human repeat, and primer adaptor sequences were then removed, leaving 382,747 sequences to form a high-fidelity (HiFi) data set. Of these, 380,352 (99.4%) aligned to human RefSeq RNA, mitochondrial, assembled chromosomes, or immunoglobulin sequences in National Center for Biotechnology Information (NCBI) databases. Of the remaining 2395 HiFi candidate sequences, one transcript (DTS1) from MCC347 cDNA aligned with high homology to African green monkey (AGM) lymphotropic polyomavirus (LPyV) and to human BK polyomavirus T antigen sequences. A second DTS transcript (DTS2) had no homology to deposited polyomavirus sequences but was subsequently identified by aligning HiFi candidates to the full-length viral genome (see below). These two sequences define a previously unknown human polyomavirus that we call Merkel cell polyomavirus (MCV or MCPyV) because of its close association with MCC.

Rapid amplification of cDNA ends (3'-RACE) extended DTS1 to three different cDNAs (Fig. 1B): One transcript terminated at a poly(A) site in the T antigen sequence, and two cDNAs read through this weak poly(A) site to form different length fusions with intron 1 of the human receptor tyrosine phosphatase type G gene (*PTPRG*) (GenBank:18860897) on chromosome 3p14.2. Viral integration at this site was confirmed by sequencing DNA polymerase chain reaction (PCR) products with the use of a viral primer and a human *PTPRG* primer. The same three RACE products were independently cloned from MCC348, a lymph node metastasis from the MCC347 primary tumor, indicating that this tumor was seeded from a single tumor cell already positive for the T antigen–PTPRG fusion transcript.

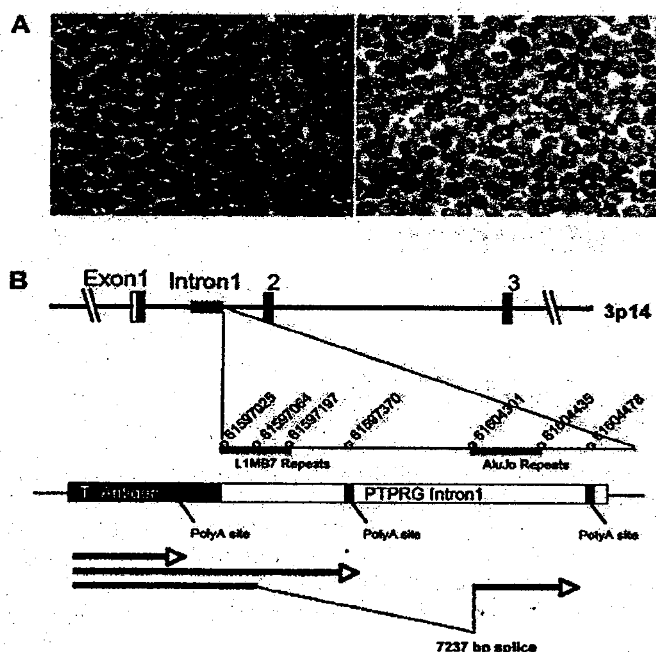


Fig. 1. (**A**) MCC is an aggressive skin cancer derived from Merkel mechanoreceptor cells that expresses neuroendocrine and perinuclear cytokeratin 20 markers, distinguishing it from other small round cell tumors (MCC349, left, hematoxylin and eosin; right, cytokeratin 20 staining, 40×. Scale bar represents 10 μm). (**B**) Discovery of Merkel cell polyomavirus transcripts in (MCC). 3'-RACE mapping of an MCC fusion transcript between the MCV T antigen and human *PTPRG*. A cDNA corresponding to a polyomavirus-like T antigen transcript was found by DTS analysis of MCC. This T antigen cDNA was extended by 3'-RACE to map three mRNA sequences (arrows), one of which terminates at a viral polyadenylation site and two of which extend into flanking human sequence and terminate in intron 1 of the human *PTPRG* gene on chromosome 3p14, indicative of viral DNA integration into the tumor cell genome. The two viral-human chimeric transcripts were generated by read-through of a weak polyadenylation signal in the viral T antigen gene. Identical RACE products were also sequenced from a lymph node metastasis of this primary tumor.

By viral genome walking, we sequenced the complete closed circular genome of MCV (5387 bp, prototype) from tumor MCC350. A second genome, MCV339 (5201 bp), was then sequenced by using MCV-specific primers. The sequences of MCV350 and MCV339 have GenBank accession numbers EU375803 and EU375804, respectively. Both viruses encode sequences with high homology to polyomavirus T antigen, VP1, VP2/3, and replication origin sequences (Fig. 2A). MCV has an early gene expression region [196 to 3080 nucleotides (nt)] containing the T antigen locus with large T and small T open reading frames and a late gene region containing VP1 and VP2/3 open reading frames between 3156 and 5118 nt. The T antigen locus has features conserved with other polyomavirus T antigens, including cr1, DnaJ, pRB1-binding Leu-X-Cys-X-Glu (LXCXE) motif, origin-binding, and helicase/adenosine triphosphatase (ATPase) domains. Mutations in the C terminus of MCV350 and 339 large T open reading frames are predicted to truncate large T protein but are unlikely to affect small T antigen protein expression. The replication origin is highly conserved with that of other polyomaviruses and includes features such as a poly(T) tract and conserved T antigen binding boxes (fig. S1). MCV has highest homology to viruses belonging to the MuPyV subgroup and is most closely related to AGM LPyV (Fig. 2B) (15). It is more distantly related to known human polyomaviruses and SV40. The principal differences between MCV350 and MCV339 are a 191-bp (1994 to 2184 nt) deletion in the MCV339 T antigen gene and a 5-bp (5216 to 5220 nt) insertion in the MCV339 late promoter. Excluding these sites, only 41 (0.8%) nucleotides differ between MCV350 and 339.

To investigate the association between MCV infection and MCC, we compared tumors from 10 MCC patients to two tissue control groups. The first control group was composed of unselected tissues from various body sites (including nine skin samples) from 59 patients without MCC (table S2). These samples were taken consecutively on a single surgical day and tested for MCV positivity with two PCR primer sets in the T antigen locus (LT1 and LT3) and one in the VP1 gene (VP1). These primers do not amplify cloned human BKV or JCV genomic DNA or SV40 genome from COS-7 cells. A second control group composed of skin and skin tumor samples from 25 immunocompetent and immunosuppressed patients without MCC were tested with LT1 and VP1 primers (table S2). Samples were randomized and tested in a blinded fashion. Southern blotting of PCR products was performed to increase sensitivity (fig. S2).

Of the 10 MCC tumors from different patients, 8 (80%) were positive for MCV sequences by PCR (Table 1 and table S1). Seven tumors showed robust amplification, and one tumor was positive only after PCR–Southern hybridization. MCC348 (metastasis from MCC347) and

Downloaded from www.sciencemag.org on March 5, 2008

MCC338 (infiltrating tumor from MCC339) were also positive. Two tumors, MCC343 and 346, remained negative after testing with 13 PCR primer pairs spanning the MCV genome. None of the 59 control tissues, including nine skin samples, was positive by PCR alone, but five gastrointestinal tract tissues tested weakly positive after PCR–Southern hybridization (8%, $P < 0.0001$, table S2). Viral T antigen sequences were recovered from three of these samples, confirming low copy number infection. Similarly, only 4 of 25 (16%, $P = 0.0007$, table S2) additional skin and non-MCC skin tumor samples from immunocompetent and immunosuppressed patients tested positive for MCV sequences (Table 2 and table S2).

To determine whether MCV DNA was integrated into the tumor genome, we examined MCC samples by direct Southern blotting without PCR amplification. When MCV DNA in MCC tumor is digested by single-cutter restriction endonucleases, such as EcoRI or BamHI, and probed with viral sequence, four possible patterns are predicted to occur: (i) if the viral DNA exists as freely replicating circular episomes, then a ~5.4 kilobase (kb) band will be present (integrated-concatenated virus will also generate a ~5.4 kb band); (ii) if MCV DNA integrates polyclonally, as might occur during secondary infection of the tumor if MCV is a passenger virus, then diffuse hybridization from different band sizes is expected; (iii) if MCV DNA integrates at one or a few chromosomal sites, then the tumors will have identical or near-identical non-5.4-kb banding patterns; or (iv) if MCV DNA integrates at different chromosomal sites before clonal expansion of the tumor cells, then distinct bands of different sizes will be present (monoclonal viral integration).

Eight of 11 MCC DNA samples (including MCC348 metastasis from MCC347) digested with either BamHI or EcoRI showed robust MCV hybridization, and these corresponded to the same tumors positive by PCR analysis with multiple primers (Fig. 3A and fig. S3). Monoclonal viral integration (pattern iv) was evident with one or both enzymes in six tumors: MCC339, 345, 347, 348, 349, and 352 (solid arrowheads). EcoRI digestion of MCC339, for example, produced two distinct 7.5- and 12.2-kb bands that would arise only if MCV is integrated at a single site in the majority of tumor cells. MCC344 and 350 bands have episomal or integrated-concatemeric bands (open arrowhead, pattern i). MCC352 has a monoclonal integration pattern (solid arrowheads, pattern iv) on BamHI digestion as well as an intense 5.4-kb band (open arrowhead), consistent with an integrated concatemer. All three tumors negative by PCR with ethidium bromide staining (MCC337, 343, and 346) were also negative by direct Southern blotting.

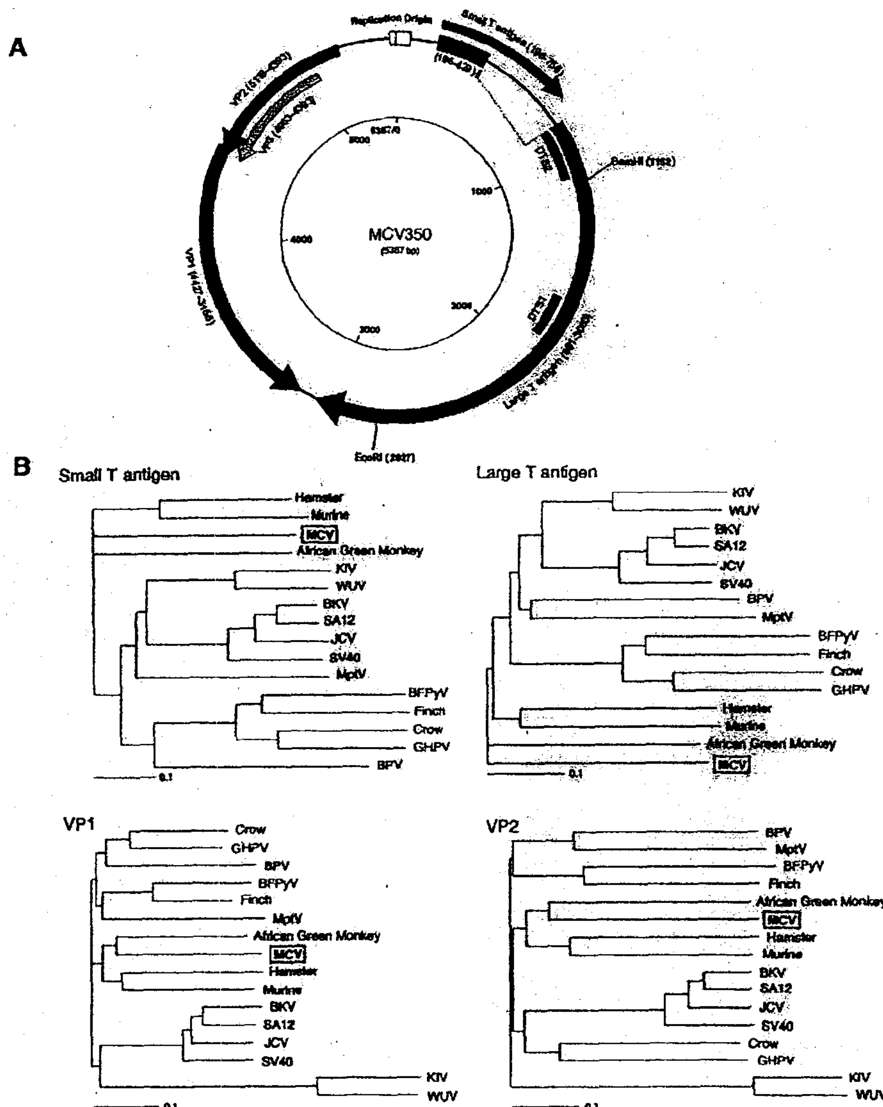


Fig. 2. **(A)** Schematic of MCV genome. Genome walking was used to clone the full MCV genome from tumor MCC350. The genome encodes typical features of a polyomavirus, including large T (purple) and small T (blue) open reading frames. Also shown are predicted VP1 (green) and overlapping VP2 (orange) and VP3 (yellow) genes. DTS1 and DTS2 (red) represent cDNA fragments originally identified by DTS screening. The former was used to identify MCV, and the latter is a spliced transcript with no homology to known polyomavirus sequences. **(B)** Neighbor-joining trees for putative MCV large T, small T, VP1, and VP2 proteins. The four known human polyomaviruses (BKV, JCV, KIV, and WUV) cluster together in the SV40 subgroup (blue), whereas MCV is most closely related to MuPyV subgroup viruses (red). Both subgroups are distinct from the avian polyomavirus subgroup (orange). Scale bars indicate an evolutionary distance of 0.1 amino acid substitutions per position in the sequence.

Table 1. PCR for MCV DNA in MCC tissues. A plus symbol indicates that the sample was strongly positive by ethidium bromide staining only with one or more primers. A minus symbol indicates that the tissue was negative for all primers. Entries with both plus and minus symbols indicate that the sample was negative by ethidium bromide staining but positive after Southern hybridization of PCR products.

	MCC cases (*n* = 10)	
Patient	**Tissue ID**	**MCV positivity**
1	MCC337	–/+
2†	MCC338	+
2	MCC339	+
3	MCC343	–
4	MCC344	+
5	MCC345	+
6	MCC346	–
7	MCC347	+
7‡	MCC348	+
8	MCC349	+
9	MCC350	+
10	MCC352	+
	Total (%)	8/10 (80)

†MCC338 was from an infiltrating tumor in skin tissue adjacent to MCC339 tumor. ‡MCC348 taken from a metastatic lymph node from MCC347.

The Southern blot banding patterns (Fig. 3A) were identical for MCC347 and its metastasis, MCC348, in line with 3′-RACE results (Fig. 1B) and confirming that MCC348 arose as a metastatic clone of MCC347. Because the genomic integration site (the *PTPRG* locus on chromosome 3p14) is mapped for these tumors, we performed Southern blotting with flanking human sequence probes to examine cellular monoclonal integration. NheI-SacI digestion of MCC347 and 348 is predicted to generate a 3.1-kb fragment from the wild-type allele and a 3.9-kb fragment from the allele containing the integrated MCV DNA. Hybridization with a flanking human *PTPRG* sequence probe revealed that the 3.9-kb allele was present in MCC347 and 348 DNA but not in control tissue DNA (Fig. 3B). As predicted, the same fragment hybridized to a MCV T antigen sequence probe, consistent with both cellular and viral monoclonality in this tumor. These results provide evidence that MCV infection and genome integration occurred in this tumor before clonal expansion of tumor cells. MCV in MCC may have some parallels to high-risk human papillomavirus (HPV), which causes cervical cancer mainly after viral episome disruption and integration into the cervical epithelial cell genome (*16*).

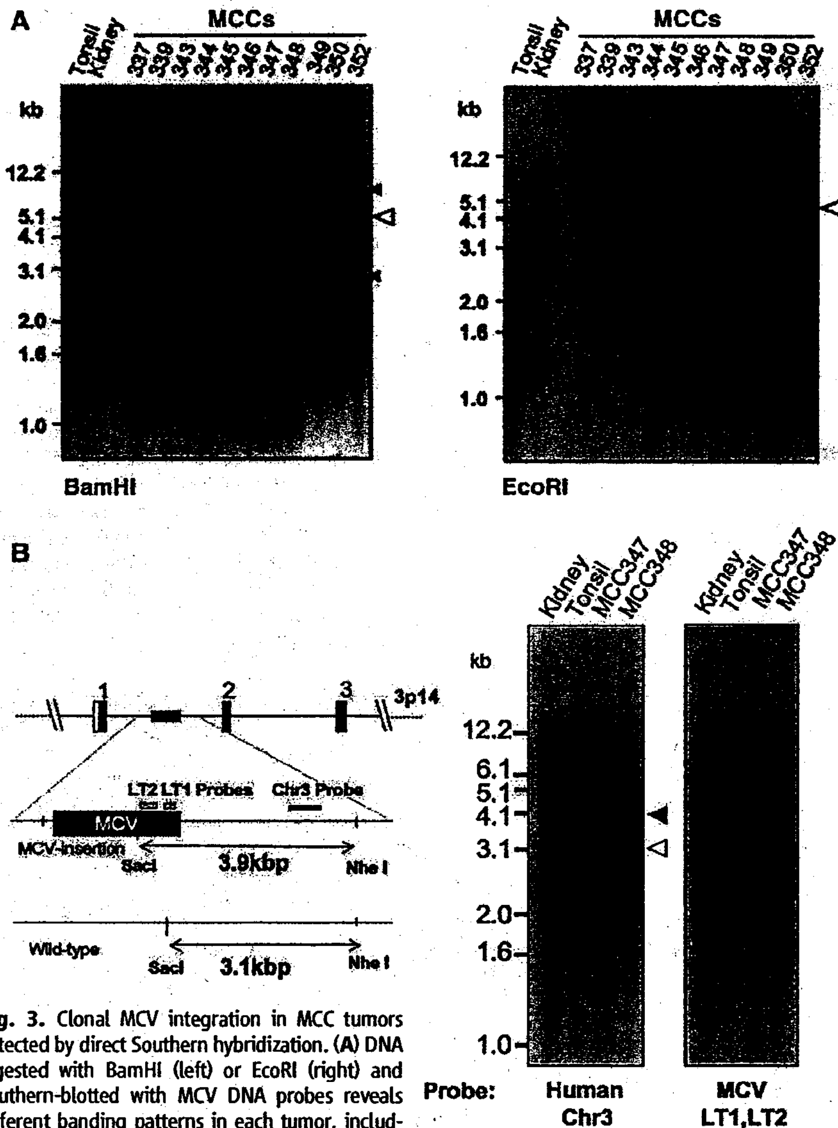


Fig. 3. Clonal MCV integration in MCC tumors detected by direct Southern hybridization. (**A**) DNA digested with BamHI (left) or EcoRI (right) and Southern-blotted with MCV DNA probes reveals different banding patterns in each tumor, including >5.4-kb bands. Open arrowhead shows the expected position for MCV episomal or concatenated-integrated genome (5.4 kb) with corresponding bands present in tumors MCC344 and 350. Tumors MCC339, 345, 347, 348, and 349 have different band sizes and doublet bands (solid arrowheads), consistent with genomic monoclonal integration. MCC352 has a prominent 5.4-kb band as well as higher and lower molecular weight monoclonal integration bands (BamHI), consistent with an integrated concatemer. Tumors MCC337, 343, and 346 have no MCV DNA detected by Southern blotting [bands at 1.5 kb (kidney) and 1.2 kb (MCC346) are artifacts]. (**B**) Viral and cellular monoclonality in MCC347 and 348. Tumor MCC347 and its metastasis MCC348 were digested with SacI and NheI and Southern-blotted with unique human flanking sequence probe [Chr3 (red), left] or viral probes [LT1 and LT2 (yellow), right]. The wild-type human allele is present in all samples at 3.1 kb (left). The MCC tumors, however, have an additional 3.9-kb allelic band formed by MCV DNA insertion into chromosome 3p14. Hybridization with probes for MCV T antigen sequence (yellow, right) generates an identical band.

If MCV plays a causal role in tumorigenesis, it could conceivably do so by several mechanisms, including T antigen expression, insertional mutagenesis, or both. Our DTS results show tumor expression of MCV T antigen, which has conserved DnaJ (*4*), pocket protein-binding LXCXE (*17*), and pp2A-binding (*18*, *19*) domains previously shown to play roles in polyomavirus-induced cell transformation. Mutational disruption of the *PTPRG* gene, which is suspected to be a tumor suppressor (*20*), could also play a role in MCC, although our Southern blot data suggest that MCV integration occurs at various genomic sites in different MCC tumors.

Our study validates the utility of DTS for the discovery of cryptic human viruses, but it has also revealed some limitations of the approach. Of the four tumors we sampled, only one (MCC347) was infected at high copy number. MCV transcripts in this tumor were present at 10 transcripts per million or about 5 transcripts per tumor cell. In future searches for other directly transforming tumor viruses (*21*), DTS should be used on multiple highly uniform samples sequenced to a depth of 200,000 transcripts or greater. Because DTS is quantitative, it is less likely to be useful in its current form for discovery of low-abundance viruses in autoimmune disorders or other chronic infectious diseases. Discovery of MCV by DTS nonetheless shows that DTS and related approaches (*22*) are promising methods to identify previously unknown human tumor viruses.

References and Notes

1. L. Gross, *Proc. Soc. Exp. Biol. Med.* 83, 414 (1953).
2. D. L. Poulin, J. A. DeCaprio, *J. Clin. Oncol.* 24, 4356 (2006).
3. S. M. Dilworth, *Nat. Rev. Cancer* 2, 951 (2002).
4. J. M. Pipas, *J. Virol.* 66, 3979 (1992).
5. K. A. Crandall, M. Perez-Losada, R. G. Christensen, D. A. McClellan, R. P. Viscidi, *Adv. Exp. Med. Biol.* 577, 46 (2006).
6. T. Allander *et al.*, *J. Virol.* 81, 4130 (2007).
7. A. M. Gaynor *et al.*, *PLoS Pathog.* 3, e64 (2007).
8. D. Hollanderova, H. Raslova, D. Blangy, J. Forstova, M. Berebbi, *Int. J. Oncol.* 23, 333 (2003).
9. B. Lemos, P. Nghiem, *J. Investig. Dermatol.* 127, 2100 (2007).
10. M. Van Gele *et al.*, *Oncogene* 23, 2732 (2004).
11. E. A. Engels, M. Frisch, J. J. Goedert, R. J. Biggar, R. W. Miller, *Lancet* 359, 497 (2002).
12. Y. Chang *et al.*, *Science* 266, 1865 (1994).
13. H. Feng *et al.*, *J. Virol.* 81, 11332 (2007).
14. H. H. Chou, M. H. Holmes, *Bioinformatics* 17, 1093 (2001).
15. M. Pawlita, A. Clad, H. zur Hausen, *Virology* 143, 196 (1985).
16. M. Durst, L. Gissmann, H. Ikenberg, H. zur Hausen, *Proc. Natl. Acad. Sci. U.S.A.* 80, 3812 (1983).
17. J. A. DeCaprio *et al.*, *Cell* 54, 275 (1988).
18. D. C. Pallas *et al.*, *Cell* 60, 167 (1990).
19. G. Walter, R. Ruediger, C. Slaughter, M. Mumby, *Proc. Natl. Acad. Sci. U.S.A.* 87, 2521 (1990).
20. D. M. Pitterle, E. M. Jolicoeur, G. Bepler, *In Vivo (Athens)* 12, 643 (1998).

Table 2. PCR for MCV DNA in comparison control tissues ($n = 84$). For detailed description of tissues and tissue sites, see table S2. MCV positivities marked with plus and minus symbols together are as in Table 1. For the various body site tissues, there were 59 samples; for the skin and skin tumor tissues, the sample size was 25 (table S2).

	MCV positivity
Various body site tissues	
Total MCV negative (%)	54/59 (92)
Total MCV positive (%)	5/59 (8)
Appendix control 1	–/+
Appendix control 2	–/+
Gall bladder	–/+
Bowel	–/+
Hemorrhoid	–/+
Skin and skin tumor tissues	
Total MCV negative (%)	21/25 (84)
Total MCV positive (%)	4/25 (16)
Skin	–/+
KS skin tumor 1	–/+
KS skin tumor 2	–/+
KS skin tumor 3	–/+

21. J. Parsonnet, in *Microbes and Malignancy*, J. Parsonnet, Ed. (Oxford Univ. Press, New York, 1999), pp. 3–18.
22. Y. Xu *et al.*, *Genomics* **81**, 329 (2003).
23. We thank the National Cancer Institute–supported Cooperative Human Tissue Network for tissues used in this study, M. Aquafondata for tissue staining, P. S. Schnable for sharing cDNA data sets used in DTS pilot testing, O. Gjoerup and R. D. Wood for helpful comments, and J. Zawinul for help with the manuscript. Supported in part by funds from NIH R33CA120726 and the Pennsylvania Department of Health. The Pennsylvania Department of Health specifically disclaims responsibility for any analyses, interpretations, or conclusions.

Supporting Online Material
www.sciencemag.org/cgi/content/full/1152586/DC1
Materials and Methods
Figs. S1 to S3
Tables S1 to S5
References

5 November 2007; accepted 8 January 2008
Published online 17 January 2008;
10.1126/science.1152586
Include this information when citing this paper.

Worldwide Human Relationships Inferred from Genome-Wide Patterns of Variation

Jun Z. Li,[1,2]*† Devin M. Absher,[1,2]* Hua Tang,[1] Audrey M. Southwick,[1,2] Amanda M. Casto,[1] Sohini Ramachandran,[4] Howard M. Cann,[5] Gregory S. Barsh,[1,3] Marcus Feldman,[4]‡ Luigi L. Cavalli-Sforza,[1]‡ Richard M. Myers[1,2]‡

Human genetic diversity is shaped by both demographic and biological factors and has fundamental implications for understanding the genetic basis of diseases. We studied 938 unrelated individuals from 51 populations of the Human Genome Diversity Panel at 650,000 common single-nucleotide polymorphism loci. Individual ancestry and population substructure were detectable with very high resolution. The relationship between haplotype heterozygosity and geography was consistent with the hypothesis of a serial founder effect with a single origin in sub-Saharan Africa. In addition, we observed a pattern of ancestral allele frequency distributions that reflects variation in population dynamics among geographic regions. This data set allows the most comprehensive characterization to date of human genetic variation.

In the past 30 years, the ability to study DNA sequence variation has dramatically increased our knowledge of the relationships among and history of human populations. Analyses of mitochondrial, Y chromosomal, and autosomal markers have revealed geographical structuring of human populations at the continental level (*1–3*) and suggest that a small group of individuals migrated out of eastern Africa and their descendants subsequently expanded into most of today's populations (*3–6*). Despite this progress, these studies were limited to a small fraction of the genome, to limited populations, or both, and yield an incomplete picture of the relative importance of mutation, recombination, migration, demography, selection, and random drift (*7–10*). To substantially increase the genomic and population coverage of past studies (e.g., the HapMap Project), we have examined more than 650,000 single-nucleotide polymorphisms (SNPs) in samples from the Human Genome Diversity Panel (HGDP-CEPH), which represents 1064 fully consenting individuals from 51 populations from sub-Saharan Africa, North Africa, Europe, the Middle East, South/Central Asia, East Asia, Oceania, and the Americas (*11*). This data set is freely available (*12*) and allows a detailed characterization of worldwide genetic variation.

We first studied genetic ancestry of each individual without using his/her population identity. This analysis considers each person's genome as having originated from K ancestral but unobserved populations whose contributions are described by K coefficients that sum to 1 for each individual. To increase computational efficiency, we developed new software, *frappe*, that implements a maximum likelihood method (*13*) to analyze all 642,690 autosomal SNPs in 938 unrelated and successfully genotyped HGDP-CEPH individuals (*14*). Figure 1A shows the results for $K = 7$; those for $K = 2$ through 6 are in fig. S1. At $K = 5$, the 938 individuals segregate into five continental groups, similar to those re-

[1]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305–5120, USA. [2]Stanford Human Genome Center, Stanford University School of Medicine, Stanford, CA 94305–5120, USA. [3]Department of Pediatrics, Stanford University School of Medicine, Stanford, CA 94305–5120, USA. [4]Department of Biological Sciences, Stanford University, Stanford, CA 94305–5120, USA. [5]Foundation Jean Dausset-Centre d'Etude du Polymorphisme Humain (CEPH), 75010 Paris, France.

*These authors contributed equally to this work.
†Present address: Department of Human Genetics, University of Michigan, 5789A MS II, Ann Arbor, MI 48109–5618, USA.
‡To whom correspondence should be addressed. E-mail: marc@charles.stanford.edu (M.F.); cavalli@stanford.edu (L.L.C.S.); myers@shgc.stanford.edu (R.M.M.)

別紙様式第2-1

No. 4

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2007. 11. 22	該当なし	
一般的名称	(製造承認書に記載なし)		研究報告の公表状況	Kato Y, Masuda G, Itoda I, Imamura A, Ajisawa A, Negishi M. J Travel Med. 2007 Sep-Oct;14(5):343-5.	公表国 日本	
販売名(企業名)	合成血「日赤」(日本赤十字社) 照射合成血「日赤」(日本赤十字社) 合成血-LR「日赤」(日本赤十字社) 照射合成血-LR「日赤」(日本赤十字社)					

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
○海外帰国者とその妻におけるブルセラ症:*Brucella melitensis*のヒト-ヒト感染の可能性 ブルセラ症は世界では一般的な人獣共通感染症で、年間50万例以上のヒト感染症例がある。ブルセラ症の一次感染は、*Brucella*種に汚染された殺菌処理されていない乳製品の摂取によって起こるが、複数の報告で男性から女性パートナーへブルセラ症が伝播した可能性が示唆されており、それらの症例は性交渉による感染と考えられてきた。 症例1:64歳の日本人男性が、6週間続く発熱で1998年6月2日に都内の病院に入院した。過去1週間の激しい腰痛も訴えた。入院時の血液培養からグラム陰性桿菌が検出され、*Brucella melitensis*バイオタイプ2と同定された。ブルセラ菌抗体価は800IUで、骨髄と肝生検からブルセラ症と確定された。患者は同年3月にイラクのバグダッドに10日間の滞在歴があり、滞在中にヒツジのチーズを摂取したことが判明した。抗生物質の投与によって症状は治まり、4ヵ月の投薬で完全に回復した。 症例2:患者1の妻で60歳の日本人女性が、1998年5月31日から発熱と左胸鎖関節の痛みを訴え始めた。血液と関節液の培養で*B melitensis*が生育した。ブルセラ菌抗体価は800IUであったが、抗生物質の投与によって回復した。患者はイラクへの渡航歴はなく、ブルセラ症に関する他のリスク要因もなかった。 考察:イラクを含め中東ではブルセラ症の発生数は多いが、日本では稀なことから、患者1は海外滞在中にブルセラ症に感染したと考えられる。2人の患者の発症には1ヵ月程度の間隔があり、標準的なブルセラ症の潜伏期間と一致する。患者1はイラクから日本に乳製品を持ち込んでおらず、患者2とブルセラ症との疫学的関連はない。患者1は疾患初期に患者2と性交渉があったことを報告しており、おそらく患者1から患者2への性感染が起こったと考えられる。同様に性感染と考えられる症例は過去にも報告されている。	合成血「日赤」 照射合成血「日赤」 合成血-LR「日赤」 照射合成血-LR「日赤」 血液を介するウイルス、細菌、原虫等の感染 vCJD等の伝播のリスク

報告企業の意見	今後の対応
イラクからの帰国者からその妻へ、ブルセラ症が性感染した可能性があるとの報告である。	日本赤十字社では、輸血感染症対策として問診時に海外渡航歴の有無を確認し、帰国(入国)後4週間は献血不適としている。また、問診で発熱などの体調不良者を献血不可としている。今後も引き続き情報の収集に努める。

Brucellosis in a Returned Traveler and His Wife: Probable Person-To-Person Transmission of *Brucella melitensis*

Yasuyuki Kato, MD, Gohta Masuda, MD, Ichiro Itoda, MD, Akifumi Imamura, MD, Atushi Ajisawa, MD, and Masayoshi Negishi, MD

Department of Infectious Diseases, Tokyo Metropolitan Komagome Hospital, Tokyo, Japan

DOI: 10.1111/j.1708-8305.2007.00139.x

Brucellosis is the most common zoonosis worldwide, with more than 500,000 new human cases annually. Although brucellosis is primarily transmitted to humans through the consumption of unpasteurized dairy products contaminated with *Brucella* species, several reports have indicated that brucellosis may be transmitted from a man to his female partner. It has been suggested that sexual intercourse is a means of transmission in these cases. Here, we describe an additional case of probable person-to-person transmission of *Brucella melitensis* in an elderly couple.

Case Report 1

A previously healthy 64-year-old Japanese man with a 6-week history of febrile illness was admitted to hospital in Tokyo, Japan, on June 2, 1998, following a 10-day visit to Baghdad, Iraq, on March 8, 1998. He also complained of severe lower back pain for 1 week. Findings on admission were fever (maximum temperature, 39.5°C) and normal pulse rate (80 beats/min). Neither heart murmurs nor adventitious breath sounds was heard. The liver was palpable 2 cm below the right costal margin; yet, the spleen was not palpated. He had tenderness of the lumber spine without abnormal neurological findings. He had no signs of epididymoorchitis. The white blood cell count was 8,400/μL and hemoglobin concentration 12.5 g/dL. Liver function tests showed elevation of alkaline phosphatase (378 IU/L) and alanine aminotransferase (67 IU/L). The erythrocyte sedimentation rate was 67 mm/h. Urinalysis findings were normal. Chest X-ray showed no opacities. T1-weighted magnetic resonance imaging of the spine revealed decreased signal intensity in the L3, L4, and L5 vertebral bodies and adjacent epidural space. These findings indicated that the patient had spondylitis, complicated by an epidural abscess.

The Gram-negative bacilli yielded by the blood culture at admission were subsequently confirmed as *Brucella melitensis* biotype 2. The *Brucella* antibody titer by the tube agglutination test was 800 IU. In addition, bone marrow and liver biopsy specimens showed evidence of granulomas consistent with brucellosis. A detailed travel history revealed that he had consumed sheep's cheese during his stay in Iraq. After confirmation of brucellosis, he was treated with intramuscular streptomycin (1 g daily), oral doxycycline (100 mg twice daily), and rifampicin (600 mg daily) for 1 month, and the fever and lower back pain gradually subsided. This treatment was followed by oral rifampicin (600 mg daily), trimethoprim–sulfamethoxazole (two standard-strength tablets twice daily), and tosufloxacin (200 mg thrice daily) for 4 months, with complete resolution.

Case Report 2

The wife of patient 1, a previously healthy 60-year-old Japanese woman, began to complain of fever and pain in the left sternoclavicular joint on May 31, 1998. Cultures of blood and the joint fluid grew *B melitensis* biotype 2. The *Brucella* antibody titer by the tube agglutination test was 400 IU. She was successfully treated with oral rifampicin (600 mg daily) and doxycycline (100 mg twice daily) for 6 weeks in combination with intramuscular streptomycin (750 mg daily) for the first 3 weeks. She did not visit Iraq with her husband and had no other risk factors for brucellosis.

Corresponding Author: Yasuyuki Kato, MD, Disease Control and Prevention Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan. E-mail: ykato@imcj.hosp.go.jp

226

© 2007 International Society of Travel Medicine, 1195-1982

Discussion

The Middle East, including Iraq, has the highest incidence of brucellosis in the world, whereas Japan is considered to be a brucellosis-free country.[1] Brucellosis is one of the reportable infectious diseases in Japan. According to the national surveillance data, only three cases of human brucellosis and two of livestock brucellosis were reported between 1999 and 2005 in Japan. No outbreaks of animal or human brucellosis were reported in Japan in 1998. Considering the incubation period of brucellosis (usually 2–4 wk, up to several months), his consumption of sheep's cheese in a brucellosis-endemic country, Iraq, and the rarity of brucellosis in his residential country, Japan, it is likely that patient 1 contracted brucellosis during his stay abroad.

The serial interval of the disease onset between patient 1 and patient 2 was approximately 1 month, which is similar to the mean incubation period of human brucellosis. Although the incubation period of brucellosis varies widely, it is difficult to argue that a common source exposure, such as food poisoning, occurred in these two patients, since patient 1 did not bring any dairy products or animals into Japan from Iraq. Furthermore, patient 2 had no other epidemiological links to brucellosis. Therefore, it is strongly suggested that the disease was transmitted from patient 1 to patient 2.

Through a PubMed search (1966–2005), we found six case reports of probable person-to-person transmission, excluding cases associated with blood transfusion, bone marrow transplantation, and breast-feeding (Table 1). Two of them are associated with international travel. In summary, it seems that men with symptoms of brucellosis are able to transmit the disease to their female partners. It is speculated that sexual transmission occurred in these cases since this is well known in animals. Interestingly, Mantur and colleagues reported that *B melitensis* was isolated from the semen, urine, and saliva of a man with epididymoorchitis, who transmitted the disease to his wife.[6] However, the presence of epididymoorchitis does not seem to be related to the transmissibility of human brucellosis. Furthermore, another report described that *B melitensis* was isolated from the sperm of one patient.[8] Patient 1 reported that he had intercourse with patient 2 during the initial stages of the disease. Therefore, we consider that person-to-person transmission, probably sexual transmission, of *B melitensis* occurred in our case.

Table 1 Published case reports of probable person-to-person transmission of brucellosis between men and women (English literature only)

Case reports	Goossens et al[2]	Stantic-Pavlinic et al[3]	Ruben et al[4]	Lindberg et al[5]	Mantur et al[6]	Thalhammer et al[7]	Present case
Age (y), sex, risk factor of primary case	25, male, laboratory exposure	34, male, laboratory exposure	61, male, laboratory exposure	35, male, travel to endemic area	30, male, animal exposure	25, male, travel to endemic area	65, male, travel to endemic area
Epididymoorchitis	Absent	Absent	Absent	Present	Present	Absent	Absent
Country where primary case was infected	Belgium	Yugoslavia	United States	Spain	India	Syria	Iraq
Age (y), sex, relationship of secondary case	21, female, fiancée	30, female, spouse	61, female, spouse	30, female, girlfriend	22, female, spouse	ND, female, girlfriend	60, female, spouse
Serial interval between two cases	3 mo	3 mo	8 mo	5 mo	1 mo	2 mo	1 mo
Isolated *Brucella* species and biotype	*Brucella melitensis* biotype 3	*B melitensis* biotype 2	*B melitensis* biotype 3	*B melitensis* biotype ND	*B melitensis* biotype 1	*Brucella abortus* biotype ND	*B melitensis* biotype 2
Suspected transmission route	Sexual intercourse	Sexual intercourse	Sexual intercourse	Sexual intercourse	Sexual intercourse	Sexual intercourse	Sexual intercourse

ND = not determined.

Although it has a little role in the epidemiology of brucellosis, person-to-person transmission is rather important in areas where brucellosis is not endemic such as most of developed countries; brucellosis has become a common imported disease in these areas.[9] Febrile-returned travelers should be educated to abstain from sexual intercourse because they could transmit the diseases to their partners. We would like to add brucellosis to the list of travel-related infections that are transmissible through sexual intercourse. This unusual mode of transmission of a common zoonosis requires special attention.

Acknowledgments

The authors thank Dr Yumiko Imada at the National Institute of Animal Health, Japan, for the identification of *B melitensis* and performance of serum agglutination tests for *Brucella*.

Declaration of Interests

The authors state that they have no conflicts of interest.

References

1. Pappas G, Papadimitriou P, Akritidis N, et al. The new global map of human brucellosis. Lancet Infect Dis 2006; 6:91–99.
2. Goossens H, Marcelis L, Dekeyser P, Butzler JP. *Brucella melitensis*: person-to-person transmission? Lancet 1983; 1:773.
3. Stantic-Pavlinic M, Cec V, Mehle J. Brucellosis in spouses and the possibility of interhuman infection. Infection 1983; 11:313–314.
4. Ruben B, Band JD, Wong P, Colville J. Person-to-person transmission of *Brucella melitensis*. Lancet 1991; 337:14–15.
5. Lindberg J, Larsson P. Transmission of *Brucella melitensis*. Lancet 1991; 337:848–849.
6. Mantur BG, Mangalgi SS, Mulimani M. *Brucella melitensis*—a sexually transmissible agent? Lancet 1996; 347:1763.
7. Thalhammer F, Eberl G, Kopetzki-Kogler U. Unusual route of transmission for *Brucella abortus*. Clin Infect Dis 1998; 26:763–764.
8. Vandercam B, Zech F, de Cooman S, et al. Isolation of *Brucella melitensis* from human sperm. Eur J Clin Microbiol Infect Dis 1990; 9:303–304.
9. Pappas G, Akritidis N, Bosilkovski M, Tsianos E. Brucellosis. N Engl J Med 2005; 352:2325–2336.

医薬品 研究報告 調査報告書

識別番号・報告回数			報告日	第一報入手日	新医薬品等の区分	機構処理欄
				2008. 2. 18	該当なし	
一般的名称	新鮮凍結人血漿		研究報告の公表状況	Bertherat E, Bekhoucha S, Chougrani S, Razik F, Duchemin JB, Houti L, Deharib L, Fayolle C, Makrerougrass B, Dali-Yahia R, Bellal R, Belhabri L, Chaieb A, Tikhomirov E, Carniel E. Emerg Infect Dis. 2007 Oct;13(10):1459-1462.	公表国 アルジェリア	
販売名(企業名)	新鮮凍結血漿「日赤」(日本赤十字社) 新鮮凍結血漿-LR「日赤」(日本赤十字社)					

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
○2003年のアルジェリアにおける50年ぶりのペスト再興 2003年6月から7月にアルジェリアOran地区においてペストの集団感染が発生した。アルジェリアでは、この疾患が50年以上報告されていなかった。腺ペスト症例18名が特定され、*Yersinia pestis*が6名から分離された。初発患者を除き、全員が回復した。標的予防的化学療法、公衆衛生、ベクターコントロールが、感染制御上重要な役割を果たした。疫学的、分子生物学的な知見から、当該期間中、現地の保菌動物の存在が強く示唆されたが、その起源(再興または再持ち込み)については特定できなかった。主要な貿易港における、今回の突然かつ予期せぬペスト再興は、国際的に重要な意味を持つ公衆衛生問題の典型的な例である。また、今回の再興は、ペスト再興の危険性が現在確認されているnatural foci(げっ歯類がペスト菌を保有する地区)に限られるものではないことも示している。	新鮮凍結血漿「日赤」 新鮮凍結血漿-LR「日赤」 血液を介するウイルス、 細菌、原虫等の感染 vCJD等の伝播のリスク

報告企業の意見	今後の対応
2003年6月から7月にアルジェリアOran地区において、50年ぶりに腺ペストの集団感染が発生したとの報告である。	日本赤十字社は、輸血感染症対策として献血時に海外渡航歴の有無を確認し、帰国(入国)後4週間は献血不適としている。今後も引き続き情報の収集に努める。

229

30

Plague Reappearance in Algeria after 50 Years, 2003

Eric Bertherat,* Souad Bekhoucha,† Saada Chougrani,‡ Fathia Razik,† Jean B. Duchemin,§ Leila Houti,‡ Larbi Deharib,‡ Corinne Fayolle,¶ Banaouda Makrerougrass,‡ Radia Dali-Yahia,† Ramdan Bellal,† Leila Belhabri,# Amina Chaieb,* Evgueni Tikhomirov,* and Elisabeth Carniel¶

An outbreak of plague occurred in the region of Oran, Algeria, from June to July 2003. Algeria had not reported this disease for >50 years. Eighteen bubonic cases were identified, and *Yersinia pestis* was isolated from 6 patients. Except for the index case-patient, all patients recovered. Targeted chemoprophylaxis, sanitation, and vector control played a crucial role in controlling the outbreak. Epidemiologic and biomolecular findings strongly suggested the existence of a local animal reservoir during this period, but its origin (resurgence or re-importation) could not be determined. This sudden and unexpected reemergence of plague, close to an important commercial seaport, is a textbook illustration of a public health event of international importance. It also demonstrates that the danger of plague reoccurrence is not limited to the currently indexed natural foci.

Plague is primarily a bacterial zoonosis affecting rodents. It is caused by *Yersinia pestis* and is transmitted from animal to animal by fleas. Humans usually become infected through the bite of an infected rodent flea. Bubonic plague, a severe infectious disease which, in the absence of appropriate antimicrobial drug therapy, can evolve to a rapidly fatal septicemia or pneumonia, can develop. A pneumonia form, which enables direct transmission to contacts, can be responsible for highly lethal outbreaks.

Currently, plague natural foci persist in Asia, the Americas, and Africa (where most human cases occur) (*1*). Plague foci have previously existed in the northern part of Africa but gradually disappeared in the last century, for unknown reasons. Libya is the only north African country that has experienced human cases in the past 40 years (*2*). In Algeria, archives report epidemics of plague as far back as the 14th century. These epidemics mainly affected ports, particularly that of Oran in 1556 and 1678 (3,000 deaths). In 1899, after an absence of nearly 100 years, plague reappeared in the port of Philippeville (now Skikda). Three large epidemics were subsequently reported in 1921 (185 cases), 1931 (76 cases), and 1944 (95 cases) as well as 158 sporadic cases. All but 2 cases occurred in ports (*3,4*). No natural focus of plague had ever been described in Algeria (*5*). We describe an outbreak of bubonic plague that occurred in 2003 in Algeria, where the last reported human case occurred in Oran in 1946 (*6*).

*World Health Organization, Geneva, Switzerland; †University Hospital, Oran, Algeria; ‡University of Medicine, Oran, Algeria; §Centre d'Etude et de Recherche pour les Meningocoques et les Schistosomiases, Niamey, Niger; ¶Institut Pasteur, Paris, France; and #Institut Pasteur, Oran, Algeria

Methods

During June 9–18, 2003, several patients with signs of severe infection and painful inflammatory adenopathy were admitted to the University Hospital of Oran. All came from Kehailia (35°29′N, 0°32′E), a village of 1,300 inhabitants 25 km south of Oran. After eliminating all other possible differential diagnoses, clinicians suspected plague. The diagnosis was confirmed on June 18 by results of analysis of a bubo (lymph node) aspirate. A technical crisis committee was set up, and a case definition was adopted (Table). Any patient with a febrile syndrome and adenopathy who resided in the prefecture of Oran was hospitalized.

Clinical samples collected from patients (blood, bubo aspirate, cerebrospinal fluid) were sent to the Microbiology Department, University Hospital, Oran. Several of the initial cases were first diagnosed with the rapid diagnostic test (RDT) for plague developed by the Institut Pasteur (*7*); however, all samples were also examined with standard bacteriologic methods. Direct examination of smears was performed after Wayson and Gram staining. Blood samples were cultured in Castaneda medium for at least 10 days

Table. Plague case definition adopted by technical crisis committee, 2003 plague outbreak, Oran region, Algeria*

Case definition	Criteria
Suspected	Clinical and epidemiologic characteristics compatible with plague; or, observation of suspect microorganisms on direct examination of clinical samples
Probable	Suspected case with anti-F1 antibodies in patient's blood; or, suspected case with a positive RDT without isolation of *Yersinia pestis* or in the absence of other cases reported in a radius of 10 km around the case
Confirmed	Culture positive for *Y. pestis*; or, RDT positive and *Y. pestis* isolated from patients living in a radius of 10 km around the case

*RDT, rapid diagnostic test.

at 28°C and examined daily. Suspected samples were inoculated into brain heart infusion and peptone broth and streaked on blood agar and cefsulodin-irgasan-novobiocin (Merck, Rahway, NJ, USA) plates. All media were incubated at 28°C. Bacterial identification was conducted with API 20 E strips (Analytab Products, Syosset, NY, USA) or individual tests in tubes. The biovar was determined (*8*). Antimicrobial drug susceptibility testing (ampicillin, amoxicillin-clavulanic acid, cefazolin, cefotaxime, gentamicin, amikacin, sulfamethoxazole, doxycycline) was conducted according to the technique of the Clinical and Laboratory Standards Institute (www.clsi.org). The serodiagnosis was determined by the ELISA-F1 technique (*9*). Serum samples from 30 study participants who had not contracted the disease but lived in the same area as the patients were used to determine the positive threshold of the technique. A serum was regarded as negative if its optical density at 490 nm (OD_{490}) was lower than a threshold defined as the mean (M) OD_{490} value of normal sera + 3 standard deviations (SD): $OD_{490} < M + 3$ SD. Sera with OD higher than this threshold were regarded as weak when the ratio $R = OD_{490}/(M + 3SD)$ was <2 and positive if R was ≥2.

Results

On June 9, 2003, a 19-year-old shepherd living in Kehailia was hospitalized with signs of septic shock (patient no. 2) (online Appendix Table, available from www.cdc.gov/EID/content/13/10/1459-appT.htm). He had been treated at home unsuccessfully with cephalosporins for inguinal adenopathy and fever during the previous 8 days. In the same village, 6 similar cases (nos. 3–8) occurred in the following days, until the diagnosis of plague was suspected and confirmed on June 18, first by RDT and then by isolation of a bacterium that had all the characteristics of *Y. pestis* biovar Orientalis and was susceptible to the antimicrobial agents tested. The epidemiologic investigation uncovered the index patient (no. 1), an 11-year-old child from Kehailia who was a cousin of case-patient 2. On June 2, an inguinal adenopathy with fever developed, and patient 2 was transferred to the hospital. He died 3 hours later, without a precise diagnosis.

Following the sanitation measures (reduction of rodent harborage, garbage removal, and vector control) implemented in Kehailia, no new cases of plague were reported in this locality after June 17. On June 19, a woman living in the suburbs of Oran (Hai Oussama) was hospitalized with bubonic plague (patient 9). The investigation showed that she had gone to Kehailia in the preceding days to consult a healer. Five cases of bubonic plague (nos. 10, 11, 14, 15, and 17) subsequently occurred from June 21 to July 16 among persons living in villages around Kehailia.

On June 28, a farmer and his wife (patients 12 and 13) who resided in Ain Temouchent, 50 km west of Kehailia (Figure), were hospitalized in Oran for symptoms suggestive of plague. The patients reported that they had not left their farm during the weeks preceding their illness. On July 1, a child from Beni Saf, on the Mediterranean coast 100 km southwest of Kehailia (Figure), had clinical signs of bubonic plague and a positive RDT result (patient 16). Neither he, nor his parents, had gone to the area of Kehailia or Ain Temouchent during the previous days. The last case (patient 18) occurred on July 22. The patient, a hunter who lived in Oran, had walked in the forest of M'sila, 30 km northwest of Kehailia, a few days before onset of his clinical signs.

Altogether, 18 cases were identified June 4–July 22, 2003: 10 confirmed, 3 probable, and 5 suspected (or 12 confirmed, 2 probable, and 4 suspected, according to the new World Health Organization case definition [*1*]). Most of the patients lived in unsanitary conditions, in close contact with livestock, and in the vicinity of storage areas of grain and fodder. In Kehailia, all the case-patients resided in different dwellings located within a 200-m radius. None of them reported direct contact with rodents. Sixteen of the 18 patients had an inguinal bubo, indicative of a flea bite on the leg. A septicemic form of plague developed in patients 1 and 2. Patient 1 died very soon after hospital admission. Patient 2 was admitted with a severe fever and neurologic syndrome and fell into a deep coma, despite broad-spectrum antimicrobial drug treatment that included vancomycin, cefotaxime, and gentamicin. He recovered from the coma 48 hours after treatment with ciprofloxacin (500 mg 2×/d for 30 days) was completed (F. Razik et al., unpub. data). No case of secondary pulmonary dissemination was observed. Other plague patients were treated with either doxycycline for adults (200 mg/d for 10 days) or cotrimoxazole for children (40 mg/kg/d for 10 days). All recovered without sequelae.

On the whole, 60 bubo aspirates, 143 blood samples, 6 sputum samples, and 2 cerebrospinal fluid samples were analyzed. In 5 samples, smear stains suggested infection

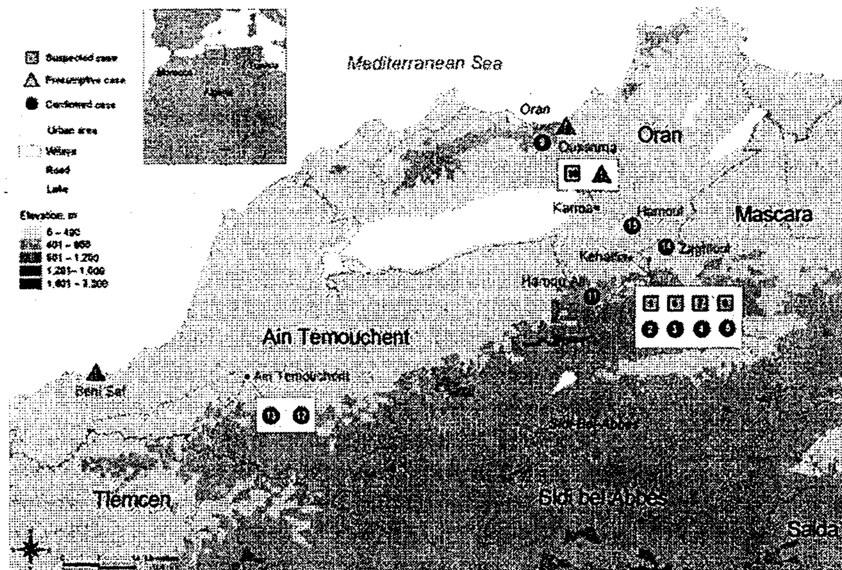


Figure. Geographic distribution of plague cases, Oran region, Algeria, June–July 2003. Boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization (WHO) concerning the legal status of any country, territory, city, or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. Data source: Ministry of Health Algeria. Map production: Public Health Mapping and GIS, Communicable Diseases, WHO. Copyright WHO, 2006. Used with permission.

with *Y. pestis* (online Appendix Table). Among the 18 patients, 12 had a positive RDT result, but *Y. pestis* was isolated from only 6 patients: 5 from bubo aspirates and 1 from the blood culture of a patient whose bubo was too small to be punctured (patient 13). Results of ELISA-F1 serologic test conducted on the serum samples from 15 of the 18 patients were strongly positive 3 times and slightly positive 3 times (online Appendix Table).

Discussion

Epidemiologic investigation did not identify any other plague patients before patient 1. It is unlikely that other cases occurred and remained undetected during this period since plague, even in its bubonic form, is a severe infection with high fatality rates.

For the first time, the RDT was used in an epidemic situation outside of Madagascar, where it was developed. The case definition had to take into account this particularity. The bacteriologic diagnosis is a long procedure (at least 4 days) and, in this epidemic context, RDT contributed to the effectiveness of the response. Of the 44 RDTs that were conducted, 12 had positive results; by contrast, culture was positive only for 6. Among the 15 patients for whom a serologic test was conducted (online Appendix Table), a specific antibody response developed only in 6. This absence of specific antibodies can be explained by the fact that serum specimens were taken before the appearance of anti-F1 immunoglobulin G, or by a rapid administration of antimicrobial drugs, which stopped development of an immune response. The 3 clearly seropositive patients were those from whom a positive culture was obtained.

The outbreak occurred in a poor rural settlement, with inadequate sanitation. The residents observed an increase in the population of commensal rodents, which is often associated with the harvesting period, but no unusual rodent mortality was noted during the weeks preceding the outbreak. The appearance during the same week of 2 new cases in Ain Temouchent (50 km west of Kehailia) and then 1 case in Beni Saf (100 km southwest of Kehailia) could not be explained. Nonetheless, the fact that the *Y. pestis* strains isolated in Kehailia and Ain Temouchent had identical pulsotypes (V. Chenal-Francisque et al., unpub. data) argues for a single focus and not for independent foci that emerged simultaneously.

A crisis committee designed and supervised a control strategy based on standardized case management, prophylactic treatment and follow-up of contacts sharing the same dwelling as plague patients, and vector control. Environmental sanitation measures in Kehailia contributed to reduction in the occurrence of new cases in this village. Intra- and peridomestic spraying with permethrin was conducted. Deltamethrin was dusted on the tracks and around the burrows of rodents located in a radius of 10 km around the dwelling of the patients. Uncontrolled killing of rats was prohibited.

No natural focus of plague had ever been described in Algeria. Past cases were always regarded as imported through the ports. The reappearance of human cases in this area can be explained in 2 ways: a recent importation of infected animals or a sudden manifestation of a natural focus that had remained silent for decades. It is noteworthy that Kehailia, the epicenter of the outbreak, is in the vicinity of flour mills built 4 years before the outbreak. These mills are supplied regularly with cereals by trucks arriving from the port of Oran. A part of this traffic was still run by railway a year before the outbreak, and a marshalling yard was installed a few kilometers from Kehailia. In 1919, this mode of importation was responsible for the plague outbreak that