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Detection and Differentiation of Human Parvovirus Variants by Commercial Quantitative Real-Time PCR Tests

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Parvovirus B19 causes a variety of diseases in humans, with outcomes ranging from asymptomatic to severe, such as chronic anemia in immunocompromised patients or fetal hydrops and death after maternal infection during pregnancy. The virus may be transmitted via plasma-derived products. According to the results of solvent-detergent safety studies, an upper limit of B19 DNA in plasma pools was recently defined. To restrict the input of B19 virus into production pools, a quantitative nucleic acid test is a prerequisite. We examined the suitability of the two commercial quantitative B19 PCR tests, LightCycler-Parvovirus B19 quantification kit (Roche Diagnostics) and RealArt Parvo B19 LC PCR (Artus) for detection, quantification, and differentiation of the three known B19 genotypes, including the newly described erythrovirus variants (genotypes 2 and 3). The former kit was highly sensitive for genotype 1 but was not suitable for detection of genotype 2 or one of two genotype 3 strains. The latter kit detected and differentiated all three genotypes, albeit with lower sensitivity for one of the genotype-3 strains. We furthermore assessed the prevalence of the three B19 virus genotypes in blood donors, by screening pooled plasma samples derived from 140,160 Finnish blood-donor units. None of the pools contained detectable levels of B19 virus genotypes 2 or 3. The origin, mode of transmission, and clinical significance of these genotypes are unknown and deserve further study. The RealArt Parvo B19 LC PCR is suitable for detection, quantification, and differentiation of all three B19 virus genotypes in molecular and clinical research.

Human parvovirus B19 causes a wide range of disease. Although the infection in immunocompetent individuals is often asymptomatic or benign, patients of certain groups are at risk of severe clinical illness. During pregnancy, the B19 virus can be transmitted from the infected mother to the fetus, sometimes leading to fetal hydrops and death (3). The virus infects erythroid progenitor cells, whereby suppression of erythropoiesis in patients with chronic hemolytic anemia (such as sickle cell anemia, thalassemia, or spherocytosis) can suddenly worsen the condition (22). In subjects with immune dysfunction, e.g., patients with human immunodeficiency virus infection or congenital immunodeficiency, or recipients of organ transplants, parvovirus infection may become chronic (14, 15).

B19 infection usually occurs in childhood, whereby >50% of adults are seropositive (6, 7). Viremia in early infection occurs with extremely high titers (up to 10^{11} to 10^{13} genome equivalents/ml) (1, 9). At this point the patients tend to be asymptomatic, and B19-infected blood donors easily go unrecognized. The high-titer viremia lasts for only about 5 to 10 days, until expression of specific antibodies. Although the virus is thought to be cleared from blood, its DNA at low levels can be detected by PCR in a declining proportion for months or, less frequently, years after infection (5, 18).

Although the B19 virus is primarily spread via the respiratory route, transmission also occurs via blood or plasma products of various kinds. High seroconversion rates and some cases of symptomatic illness have been due to blood products prepared from B19-containing plasma pools (17, 30, 31). The icosahedral nonenveloped B19 virion is resistant to ordinary physicochemical factors, including solvent-detergent treatment. Because of its minute size, the pathogen is also relatively resistant to filtration (26), although it is in part removable with small-pore-size filters (4, 28). By PCR screening it is possible to restrict the DNA load and obtain safe plasma products (28).

Plasma pools also contain antibodies. However, in solvent-detergent plasma safety studies (16), the recipients were shown to seroconvert due to pools containing B19 DNA in high titers (10^7 IU/ml). These patients also became B19 DNA positive, verifying virus transmission. In contrast, transfusion of B19 DNA in concentrations of $<10^4$ IU/ml is not considered to result in detectable seroconversion, with some exceptions (2). Consequently, according to the revised European Pharmacopoeia (8), plasma pools used for human anti-D immunoglobulin production, must not (after 1 January 2004) contain $>10^4$ IU/ml of B19 DNA. In order to identify such high-titer units, a quantitative DNA detection method is required. On the other hand, new variants of the B19 virus have been discovered (13, 19, 20, 21, 25), leading to segregation of the species into three distinct genotypes (25) diverging from each other in sequence by >10%. In consequence, B19 PCR methods might fail to detect such variants due to sequence divergence between primers and viral target DNA.

We examined the suitability of the two commercial quantitative B19 PCR tests on the global market, LightCycler-Parvovirus B19 quantification kit (Roche Diagnostics GmbH, Mannheim, Germany), hereafter referred to as qPCR-1, and

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RealArt Parvo B19 LC PCR (Artus GmbH, Hamburg, Germany), hereafter referred to as qPCR-2, for the detection, quantification, and differentiation of the three B19 genotypes known: genotype 1 (prototype), genotype 2 (LaLi, A6), and genotype 3 (V9, D91.1). We furthermore assessed the prevalence of all three genotypes among Finnish blood donors by screening plasma pools derived from 140,160 blood donor units.

MATERIALS AND METHODS

Plasmids. Several plasmid constructs carrying B19 virus DNA of the different genotypes were used. Genotype 1 was represented by pB19-Lit, a near-full-length fragment of the B19 virus genome (B19-NAN) cloned into plasmid Litmus29 (New England Biolabs, Beverly, Mass.) (L. Kakkola et al., unpublished data). Genotype 2 was represented by four cloned PCR products of the LaLi sequence (GenBank accession number AY044266): pLaLi1 covering nucleotides (nt) 1 to 1189 (corresponding to nt 144 to 1340 of B19 Au, GenBank accession number M13178 [24]) in Litmus29, pLaLi2 covering nt 969 to 2209 (nt 1120 to 2361 of Au) in Litmus29, and pLaLi3 covering nt 1935 to 2998 (nt 2086 to 3149 of Au) in pSTBlue-1 (Novagen, Madison Wis.), as well as pLaLi6 that covers all three regions (nt 144 to 3149 of Au). Genotype 3 was represented by pV9-C22, a near-full-length genome of isolate V9 (GenBank accession number AJ249437), cloned in a modified plasmid pcDNA2.1 (Invitrogen Life Technologies, Paisley, United Kingdom), and pD91.1-C10, a near full-length genome of isolate D91.1 (GenBank accession number AY083234), cloned in plasmid pcDNA3.1HisB (Invitrogen).

DNA concentration of the genotype 1-containing plasmid, pB19-Lit, was first measured by the validated (K. Hokynar et al., unpublished data) qPCR-1 test. The DNA concentrations of all plasmids were then measured spectrophotometrically and converted into plasmid copy numbers per microliter. The plasmid preparations were equalized to contain 10^9 copies/μl each. To confirm the concentrations, serial dilutions of the equalized plasmid purifications were dot blotted onto nylon membrane and hybridized with a digoxigenin-labeled probe against the plasmid backbone. The plasmids were subsequently used for evaluation of the two qPCR methods.

Primer sequences for both qPCR-1 and qPCR-2 assays are considered confidential by the manufacturers. Therefore, it was necessary to map the approximate target regions for each PCR assay in order to select, among plasmids containing parts of genotype 2, those containing the appropriate target sequence. For this purpose, the amplicons of B19 genotype 1 produced by qPCR-1 and qPCR-2 were analyzed on an agarose gel, transferred to a nylon membrane, and hybridized with digoxigenin-labeled probes specific for two different genomic regions, a 359-bp fragment of the NS1 gene (12) and a 354-bp fragment of the VP1 gene (27). Hybridization of the qPCR-1 amplicon gave a positive result with the NS probe and a negative result with the VP1 probe. Since the amplicon size of qPCR-1 is ca. 170 to 180 bp and the overlapping area (covered by the NS probe) of the inserts in plasmids pLaLi1 and pLaLi2 is 223 nucleotides, both of these plasmids contain the complete target sequence of qPCR-1, and can be used as genotype 2 template in qPCR-1 (as can plasmid pLaLi6 containing the full NS region). Southern hybridization of the qPCR-2 amplicon gave a positive result with the VP1 probe and a negative result with the NS probe. Since the VP1 probe covers the VP1 unique region included in plasmid pLaLi3, this plasmid can be used as genotype-2 template sequence in qPCR-2.

Clinical samples. To further examine the suitability of the PCR kits for identification of the three genotypes in natural condition, clinical samples previously shown to contain genotype 1 (four skin biopsies), 2 (six skin biopsies), or 3 (two serum samples) were analyzed (13, 19, 25). DNA was purified by phenol-chloroform extraction, followed by ethanol precipitation. As pointed out by the manufacturer, phenol-based extraction is not recommended for qPCR-2. Therefore, to detect possible DNA polymerase inhibitors in these preparations, an internal control (IC) provided in each kit was added to the master mix (0.5 μl/reaction) and detected on channel F3. Two dilutions of each sample were studied in one to four replicates.

Blood donor samples. All blood donations collected at the Finnish Red Cross Blood Service since January 2002 were prescreened for B19 DNA by qPCR-1 as EDTA-plasma maxipools of 480 U each. Nucleic acid eluates of 292 such maxipools, representing 140,160 blood donation units altogether, were randomly chosen for comparative studies with qPCR-2. For further genotyping, all eluates positive with qPCR-2 were studied with the conventional, previously described nested-PCR methods: VP1-PCR (27) has been shown to detect genotypes 1 and 2 but not genotype 3, and K71-PCR detects only genotype 2 (13).

In addition, of 13 donations of blood with high-titer B19 DNA during 2002 and 2003, 11 were still available for genotyping. DNA was extracted in parallel by using phenol-chloroform, followed by ethanol, and by one of the recommended methods, the QIAamp DNA Minikit (Qiagen GmbH, Hilden, Germany). Both extracts were studied with qPCR-2, and the B19 genotype was determined by melting curve analysis and by the conventional VP1-PCR and K71-PCR assays.

Quantitative PCR methods. We compared the performances of two LightCycler-based qPCR assays for B19 DNA: qPCR-1 (Parvovirus B19 quantification kit by Roche) and qPCR-2 (RealArt Parvo B19 LC PCR by Artus).

The principle in both assays is basically the same. Each provides B19-specific primers and two hybridization probes labeled with fluorescent molecules. Hybridization leads to fluorescence resonance energy transfer between the two fluorophores, and the emitted light is measured by a LightCycler instrument on channel F2/Back-F1. Real-time (during amplification) monitoring of fluorescence intensities, relative to external standards of known target concentrations, allows for quantification of the accumulating product. To monitor the efficiencies of the nucleic acid extraction and the PCR process, ICs are amplified with the same primers as the target but hybridized with probes carrying different fluorophores. The fluorescence emitted from the IC-specific probes is measured on channel F3/Back-F1.

The raw data created by either qPCR test were analyzed with LightCycler Software version 3.5 (Roche). Crossing points and calculated concentrations were obtained by the second derivative maximum method together with proportional baseline adjustment. This method calculates the fractional cycle number of the crossing-point value of each sample automatically and thus makes the method independent of user-related influences. A previously generated color compensation file was activated during the LightCycler run to reduce flow-through signal from other channels.

In order to determine the sensitivity of the two B19 qPCR tests, qPCR-1 and qPCR-2, 10-fold dilutions were used of the plasmid constructs containing DNA inserts from each of the three genotypes. With both qPCRs, each dilution was tested in one to three replicates. The PCRs were performed as recommended by the manufacturer. In every PCR run, water was included as a negative control. The WHO International Standard for Parvovirus B19 DNA nucleic acid assays (NIBSC 99/800) (23) was used as a positive control. The background levels of plasmids Litmus29, pSTBlue-1, and pcDNA2.1 without insert were studied by using the plasmid backbones without inserts.

Melting curve analysis. The ability of qPCR-1 and qPCR-2 to differentiate the amplicons obtained from DNA of different B19 genotypes was examined by melting curve analysis (segment 1 [95°C; hold time, 15 s; slope, 20°C/s; acquisition mode, none], segment 2 [40°C; hold time, 15 s; slope, 20°C/s; acquisition mode, none], and segment 3 [80°C; hold time, 0 s; slope, 0.1°C/s; acquisition mode, continuous]) subsequent to the kit-specific procedures. Melting points were determined with serial dilutions of DNA from each genotype alone, as well as with serial dilutions of DNA mixtures of two or three genotypes in equal amounts.

RESULTS

qPCR of B19 DNA in plasmid form. qPCR-1 was highly sensitive for genotype 1 DNA detection (Fig. 1). A positive signal was obtained even with a theoretical load of 0.5 copy/reaction. Quantification of genotype 1 was accurate down to 50 copies/reaction. However, this method barely recognized genotype 2 (Fig. 1): in two out of four parallel runs, a positive signal was obtained only with >5 million copies of pLaLi1/reaction. Higher dilutions remained negative in every run. The qPCR-1 assay was tested further with plasmid pLaLi2 and plasmid pLaLi6 containing the full NS region. The results obtained with pLaLi2 and pLaLi6 were similar to those obtained with pLaLi1 (data not shown). Of the two genotype three isolates, only V9 was recognized, with a sensitivity of ~1 log lower than for genotype 1 (Fig. 1). The signal for the genotype 3 isolate D91.1 remained negative even with 5×10^9 copies/reaction (Fig. 1). Melting curve analysis of qPCR-1 products showed the same two peaks (63.27 and 69.5°C) for all

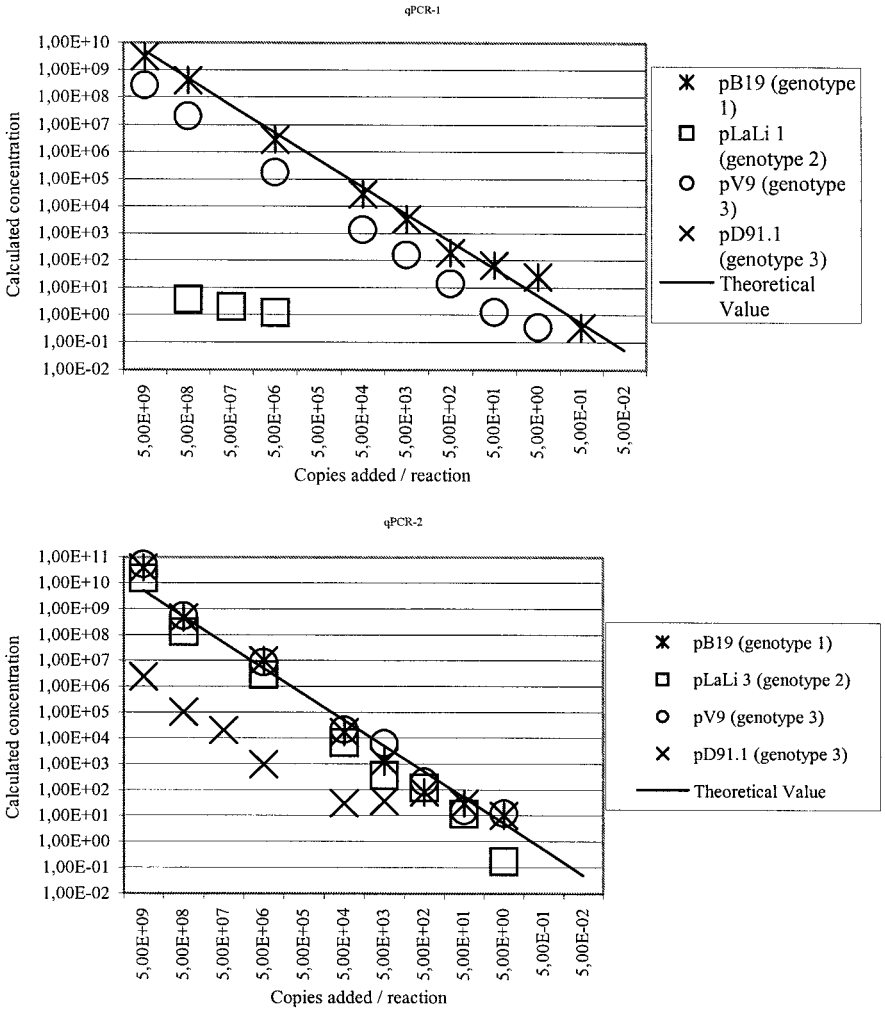

FIG. 1. Sensitivity of qPCR-1 (upper panel) and qPCR-2 (lower panel) for the different B19 virus genotypes.

amplicons regardless of the genotype (data not shown). Thus, the three B19 genotypes could not be differentiated from each other by melting-curve analysis after qPCR-1.

qPCR-2 recognized genotypes 1 and 2 and isolate V9 of genotype 3 with equal sensitivity (Fig. 1). All three were detected in copy numbers down to 5/reaction. However, the detection sensitivity for genotype 3 isolate D91.1 was ~3 log lower, with a detection limit of 5,000 copies/reaction.

The qPCR-2 amplicons of all three genotypes were well distinguished in melting-curve analysis, which upon addition of one genotype per reaction gave distinct peaks for each genotype (Fig. 2, top panel). The T_m for genotype 1, genotype 2, and both subtypes of genotype 3 were 67.57 (range, 66.53 to 68.45), 65.34 (range, 64.72 to 65.77), and 60.69 (range, 59.94 to 61.34) °C, respectively.

However, when the genotypes were tested in combinations of two or three genotypes per reaction, mimicking a mixed infection, the individual melting peaks were not always seen (Fig. 2, lower panel). A mixture of all three genotypes resulted in only two peaks characteristic of genotypes 1 and 3. If two genotypes were present in one sample, genotypes 1 and 2 showed only one peak (characteristic of genotype 1) and were not distinguished by this method, whereas both other genotype combinations, genotypes 1 and 3, as well as genotypes 2 and 3, showed the correct two peaks. As shown by plasmid dilution series, the corresponding melting points were independent of DNA load (Fig. 2). The WHO International Standard (standard 99/800) that was used as positive control, showed a single melting peak of 67.5, which is characteristic of genotype 1 (data not shown).

qPCR of B19 DNA in clinical samples. To examine the suitability of the qPCR tests for identification of the three genotypes in natural condition, clinical samples previously shown to contain genotypes 1 (four skin biopsies), 2 (six skin biopsies), or 3 (two serum samples) were analyzed either with qPCR-1 or qPCR-2 or both (Table 1). Because of the low B19 DNA level in these samples and because of the presence of inhibitory compounds in the phenol-chloroform extracted DNA preparations, as indicated by the absence of an IC signal in some of the replicates, both undiluted and 1:10-diluted samples needed to be tested.

qPCR-1 recognized two of two skin samples containing B19 DNA of genotype 1. All five skin samples containing genotype 2 and both serum samples containing the two different sub-

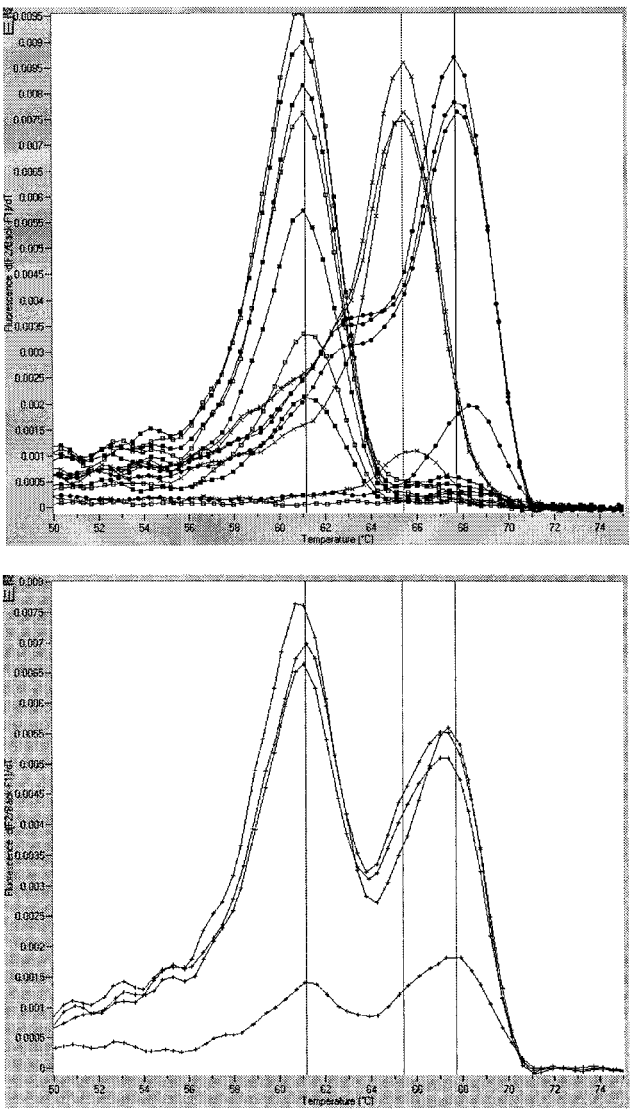

FIG. 2. Melting peaks generated by qPCR-2. The four clones were tested in copy numbers of 5×10^9, 5×10^6, 5×10^4, and 5×10^1 as one genotype per reaction (pB19-Lit [genotype 1], ●; pLaLi3 [genotype 2], ×; pV9-C22 [genotype 3], ■; and pD91.1-C10 [genotype 3], □) (upper panel) and as mixtures of the three genotypes (pB19-Lit, pLaLi3, and pV9-C22) (lower panel).

types of genotype 3 remained negative, even though the IC gave a positive signal, indicating absence of PCR inhibition.

In agreement with the results obtained for plasmid templates, qPCR-2 recognized all genotypes in clinical samples: genotype 1 in four of four skin samples, genotype 2 in 6 of 6 skin samples, and genotype 3 subtype D91.1 in one of one serum sample. As with qPCR-1, the serum containing isolate V9 of genotype 3 remained negative even though IC was positive. All of the three genotypes in the clinical samples could be differentiated by melting curve analysis.

qPCR of B19 DNA in blood-donor samples. Of the 857 maxipools prescreened by qPCR-1 at the Finnish Red Cross Blood Service, 292 were randomly selected and studied with qPCR-2 (Table 2). The samples could be divided in 4 groups: 13 of 292 (4.5%) were qPCR positive with both tests (sample group 1), 3 of 292 (1.0%) were qPCR-1 negative but qPCR-2 positive (group 2), and 51 of 292 (17.5%) were qPCR-1 positive but qPCR-2 negative (group 3). The majority of the maxipools, 225 of 292 (77.1%), were negative with both qPCRs (group 4). To verify the qPCR-2 genotype identification in each maxipool, samples positive with qPCR-2 were studied further with the conventional PCRs, VP1-PCR and the genotype 2-specific K71-PCR. The results are shown in Table 2.

Within group 1—as determined by the qPCR-1 crossing-point value—the B19 DNA load was $>10^5$ IU/ml in one sample, 10^4 to 10^5 IU/ml in two samples, 10^3 to 10^4 in four samples, and $<10^3$ IU/ml in six samples. Of these 13 samples, 7 were positive by VP1-PCR and all were negative by K71-PCR. By melting curve analysis after qPCR-2, most samples (9 of 13) gave results corresponding to genotype 1. However, four samples gave melting points of ~63.6°C, i.e., intermediary between the melting points characteristic for genotypes 2 and 3.

The three qPCR-1-negative samples in group 2 gave a weak positive signal (barely showing a curve) with qPCR-2 and were completely negative by VP1-PCR and K71-PCR. The melting point for one of these three samples was positioned at ~63°C like the four samples in group 1, and another sample showed a melting point typical for genotype 1 (68°C). No melting point could be observed for the third sample. The five samples of groups 1 and 2 with unusual melting points were retested with qPCR-2 with reproducible results. The qPCR-2 test gave concentration values for seven more qPCR-1-negative samples, which were, however, considered negative due to the lack of

TABLE 1. PCR results for the clinical samples[a]

Genotype	qPCR-1 assay			qPCR-2 assay		
	Sample (dilution)	Concn (copies/reaction)	IC signal	Concn (IU/μl)	IC signal	T_m (°C)[a]
1	A	ND		1.22E−01	−	66.18
	A (1:10)	1.64E+02	+	1.07E+02	+	67.39
	B	ND			−	
	B (1:10)	1.05E+02	+	4.30E+01	+	67.42
	C	ND		5.16E+01	−	67.73
	C (1:10)	ND			−	67.70
	D	ND			−	
	D (1:10)	ND		1.30E+01	+	67.76
2	E		+	1.08E+01	+	65.44
	E (1:10)		+	6.98E+00	+	65.53
	F		−		−	
	F (1:10)		+	9.49E+00	+	65.45
	G	ND		2.89E+00	+	65.29
	G (1:10)	ND			+	
	H		−		−	
	H (1:10)		+	4.54E+01	+	65.46
	I		+			
	I (1:10)		+	2.60E+01	+	65.41
	I (1:100)				+	
	J		−			
	J (1:10)		+	1.25E+01	+	65.3
	J (1:100)	ND			+	
3	K		+			
	K (1:100)		+			
	L		−	8.27E+04	+	60.34
	L (1:100)		−	1.71E+03	+	60.43

[a] T_m, temperature at which 50% of the double-stranded DNA strands are denatured (melting point). ND, not determined. The presence (+) or absence (−) of IC signal is as indicated.

TABLE 2. PCR results for 292 blood donor samples[a]

Group	Sample(s)	qPCR-1 assay		qPCR-2 assay		Positive (+) or negative (−) assay result	
		Crossing point[a]	Concn (log IU/ml)[b]	Concn (IU/μl)[c]	T_m (°C)[d]	VP1-PCR	K71-PCR
1	1	29.84	4–5	1.495E+01	63.66	+	−
	2	31.80	3–4	1.378E+01	63.72	+	−
	3	30.58	4–5	1.73E+01	67.77	+	−
	4	34.92	<3	1.53E+02	67.59	−	−
	5	35.64	<3	9.068E+00	67.51	−	−
	6	23.85	>5	3.87E+03	66.67	+	−
	7	34.62	<3	5.007E+00	63.27	−	−
	8	37.51	<3	1.123E+01	67.59	−	−
	9	33.41	3–4	7.553E+00	63.60	−	−
	10	33.83	3–4	2.544E+00	67.32	+	−
	11	31.68	3–4	8.672E+00	67.52	+	−
	12	34.73	<3	1.44E+01	67.65	+	−
	13	34.21	<3	3.91E+00	68.02	−	−
2	1	Neg[e]		2.891E+00	63.58	−	−
	2	Neg		3.413E+02		−	−
	3	Neg		2.58E+01	68.03	−	−
3	1–50	35.52 (34.35–38.5)	<3			ND[f]	ND
	51	33.51	3–4			ND	ND
4	1–225	Neg		Neg		ND	ND

[a] Crossing point is the fractional cycle number at which the program indicates the fluorescence of a particular specimen to rise above background levels.
[b] Level of B19 DNA in maxipool samples, estimated by using the crossing point value.
[c] Concentration of B19 DNA in maxipool samples, as determined by the kit instructions.
[d] T_m, temperature at which 50% of the double-stranded DNA strands are denatured (melting point).
[e] Neg, negative result.
[f] ND, not determined.

any visible amplification (or melting) curves. Among the 51 samples of group 3 (positive by qPCR-1 alone) the B19 DNA loads were $<10^3$ IU/ml in 50 samples and 10^3 to 10^4 IU/ml in one sample. All samples had been prescreened with qPCR-1 soon after collection (nonfrozen), and had been stored frozen at −20°C (with two thawings) before qPCR-2 testing. The reason for the negative results of qPCR-2 in group 3 might thus be degradation of low-level B19 DNA.

In addition to groups 1 to 4 in Table 2, 11 blood donations excluded from manufacture for excessive B19 DNA levels by qPCR-1 were tested and found to be positive by qPCR-2 (data not shown). The QIAamp DNA Minikit-purified samples showed a DNA concentration slightly (≤1 log) higher than the same samples extracted by phenol-chloroform. When the phenol-chloroform-extracted samples were tested diluted 1:100 in PCR-grade water, the melting points for all samples except one were characteristic for genotype 1, with an average T_m of 67.41°C (67.23 to 67.51). With undiluted samples the melting points were unusually low, i.e., 64.9°C (61.81 to 65.97). However, the melting point for one sample (sample 7) remained between the positions of genotypes 2 and 3 (T_m ~63°C) even after dilution. When DNA of these same samples was extracted with QIAamp DNA Minikit, all showed melting points typical for genotype 1 even undiluted, except plasma sample 7, which gave the unusual melting point of 63°C with this extraction method as well.

DISCUSSION

Although the B19 virus has been known for many years, its transmission to recipients of plasma derivatives despite attempted inactivation has recently reinforced the necessity of plasma pool screening. After the estimation of a "safe level" of B19 virus DNA in manufacture (16), most plasma pool fractionators have begun to quantify B19 DNA by nucleic acid testing to ensure a viral load of $<10^4$ IU/ml. Two quantitative PCR methods are commercially available and are mainly used for plasma pool screening at various sites in Europe. In the present study, qPCR-1 was slightly more sensitive than qPCR-2 in detection and quantification of B19 genotype 1. In the detection and quantification of B19 genotypes 2 and 3, however, qPCR-2 performed better: it detected all three genotypes, although it detected isolate D91.1 of genotype 3 with lower sensitivity. In contrast, qPCR-1 detected no genotype 2 and only one isolate (V9) of genotype 3. Within the current guidelines of the B19 DNA safety levels in plasma, the qPCR-2 test, after proper validation, can be recommended for prescreening of pools containing any known B19 virus genotype.

Even though an increasing number of studies have addressed the role of the B19 virus prototype (genotype 1) in transfusion of blood and plasma derivatives, knowledge on the frequency and magnitude of the variant genotypes is scanty. A main reason has been the lack of suitable tools for the simultaneous detection and distinction of all genotypes. The first B19 variant was "V9," discovered by conventional PCR in the serum of a patient with transient aplastic anemia (19). Recently, other B19 virus variants (K71 and A6) were reported by us and others (13, 21, 25), and a nomenclature based on three genotypes has been suggested (25). Due to DNA sequence divergence, most B19 PCR methods are unlikely to detect these variants. The same may hold for putative additional B19

genotypes, the detection of which may call for new methods. As shown here, qPCR-2 detected all three genotypes in clinical samples and identified them correctly by melting curve analysis. This method in principle allows for differentiation of specific from nonspecific PCR products such as primer-dimers or mispriming products, but can also be used for mutation analysis. Thereby, rapid detection and differentiation of the three genotypes in a single reaction without laborious and tedious restriction enzyme analyzes or electrophoretic procedures make qPCR-2 a valuable tool for rapid genotyping. Moreover, this method might be suitable for detection of additional genotypes. As pointed out, new B19 variants could theoretically exist in human tissues. Indeed, upon examination of our plasma pools for genotypes 2 and 3, five pools showed unusual melting points (T_m ~63°C). The same phenomenon was seen in one of the blood donations containing B19 DNA in high titer. The fact that most of these samples gave positive results also with qPCR-1 and/or VP1-PCR suggests that these atypical melting points might be due to yet another novel genotype. Further examination and sequencing of these samples is ongoing.

Previously, Heegaard et al. (11) described a method for detection of both the B19 prototype and the V9 variant. This method consisted of consensus primers for amplification of both strains, and two pairs of nested primers: one specific for the prototype and the other specific for the variant. Using this method 100 sera containing B19 immunoglobulin M and 50 blood-donor plasma pools were screened without detection of V9 (11). The same result came from 190 bone marrow samples of healthy subjects (10).

Servant et al. (25) performed PCR with primers allowing for simultaneous amplification of the B19 prototype (genotype 1), LaLi-like sequences (genotype 2) and V9-like sequences (genotype 3). Restriction enzyme analysis of the PCR product was used for initial differentiation of genotype 1 from genotypes 2 and 3, and the definitive result was obtained by sequencing. To evaluate the genotype circulation and frequency among patients of different groups, Servant et al. screened 1,084 clinical samples of different types. Indeed, LaLi-like sequences were found in two sera and V9-like sequences in nine samples (five serum, three bone marrow, and one blood sample), including subtype D91.1 detected in serum. By the same method, Nguyen et al. (21) examined 149 serum and 18 bone marrow samples. Of the 29 PCR-positive sera, only one (A6) was of genotype 2, and all of the others of genotype 1. No atypical sequences were detected in bone marrow. Upon reexamination of the Danish blood donor plasma pools (10) or 12 additional pools, merely genotype 1 was found. However, genotypes 2 and 3 were found in 5 of 83 and 2 of 83 livers, respectively (29).

We examined by qPCR-2 the prevalence of genotypes 1, 2, and 3 among Finnish blood donors. None of the 140,160 blood donations studied contained detectable levels of B19 genotypes 2 or 3. This result is in concordance with those of Heegaard et al. and suggests that viremic infections by the new genotypes, at present, are extremely rare. However, in our recent study (13) genotype 2 DNA occurred in the skin of 47% of constitutionally healthy B19-seropositive adults: a tissue genoprevalence higher than that observed with the prototype virus. Indeed, the epidemiology and clinical significance of the new B19 virus genotypes are to a large extent unknown, whereby more studies on this captivating issue are warranted.

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