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ORIGINAL ARTICLE VIROLOGY

Investigation of human parvovirus B19 occurrence and genetic variability in different leukaemia entities

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Abstract

Human parvovirus B19V (B19V) has been associated with various haematological disorders, but data on its prevalence in leukaemia are scarce. In this cross-sectional study, we investigated patients in Sao Paulo, Brazil with leukaemia to determine the molecular frequency of B19 variants and characterize the viral genetic variability by partial and complete sequencing of the coding of non-structural protein 1 (NS1)/viral capsid proteins 1 and 2 (VP1/VP2). The presence of B19V infections was investigated by PCR amplification of the viral NS1 gene fragment and confirmed by sequencing analysis. The NS1/VP1/VP2 and partially larger gene fragments of the NS1-positive samples were determined by overlapping nested PCR and direct sequencing results. The B19V NS1 was detected in 40 (16%) of 249 bone marrow samples including 12/78 (15.4%) acute lymphoblastic leukaemia, 25/155 (16.1%) acute myeloid leukaemia and 3/16 (18.7%) chronic myeloid leukaemia samples. Of the 40 participants, 25 (62.5%) were infected with genotype 1a and 15 (37.5%) with genotype 3b. The phylogenetic analysis of other regions revealed that 12/40 (30%) of the patients with leukaemia were co-infected with genotypes 1a and 3b. In addition, a new B19V intergenotypic recombinant (1a/3b) and an NS1 non-recombinant genotype 1a were detected in one patient. Our findings demonstrated a relatively high prevalence of B19V monoinfections and dual infections and provide, for the first time, evidence of inter-genotypic recombination in adults with leukaemia that may contribute to the genetic diversity of B19V and may also be a source of new emerging viral strains with future implications for diagnosis, therapy and efficient vaccine development.

Keywords: Coinfection, genotypes, human parvovirus B19, leukaemia, prevalence
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Introduction

The human parvovirus B19 (B19V) belongs to the genus Erythrovirus of the family Parvoviridae and is a tiny non-enveloped virus with linear single-stranded DNA genome. The virus propagates in actively dividing erythroid lineage progenitors of bone marrow (BM) cells, inhibiting erythropoiesis [1]. The viral genome encodes three main proteins: the non-structural protein (NS1) and two viral capsid proteins (VP1 and VP2) [2]. The minor capsid protein, VP1, has the same amino acid sequence as VP2 plus an additional 227 amino acids at the N-terminus: the VP1-unique region (VP1u) [3]. In addition to NS1 and VPs, the B19V genome also encodes a 7.5-kDa protein, an 11-kDa protein and the putative X protein. Currently, the exact function of the 7.5-kDa protein during B19V infection is not known but the 11-kDa protein has been shown to regulate virion production in infected cells [4].

Although a single-stranded DNA virus, the B19V mutation rate is higher than the rates observed in DNA viruses, and the virus may evolve at rates similar to those of many RNA viruses [5,6]. The current views of B19V diversity are shaped by partially and completely sequenced viral genomes representing three genetically distinct genotypes with more than 10% genetic

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variability among them [7–10]. These have been designated genotype 1 (B19-related viruses), genotype 2 (A6-related viruses), and genotype 3 (V9-related viruses). Furthermore, recent phylogenetic analyses have revealed two subgroups within genotypes 1 and 3 [5,11]. Most infections are caused by genotype 1 and result in a wide range of human diseases, including erythema infectiosum in children, arthropathy, transient aplastic crisis in patients with chronic haemolytic anaemia, and hydrops fetalis in pregnant women [2,12]. B19V have been increasingly proposed as responsible pathogens for acute leukaemia because of their known erythrocyte tropism, which makes them attractive and biologically plausible aetiological agents [13–17]. One report suggested that the transformation of a pre-leukaemic clone to a fully qualified malignant clone by B19V is probably attributed to the virus-induced immune suppression of erythroid elements in the BM, along with cell proliferation and up-regulation of key mediators, such as circulating granulocyte–macrophage colony-stimulating factor, which have previously been detected at abnormally high concentrations at the onset of acute leukaemia [14,18,19]. Another study has reported that B19V infections may impact leukaemia by altering DNA methylation patterns and specific key genes in susceptible cells [20,21]. Much information on the proposed causal association of B19V infection and leukaemia has been based on direct detection of viral genomes from the BM of patients with leukaemia [14,21]. However, B19V DNA has also been found in BM from healthy donors [22,23], although the number of subjects investigated was small. These findings leave open the question of whether B19V is the sole culprit or merely an innocent bystander in the BM as a consequence of an earlier infection. Generally, patients with malignant blood conditions, particularly children with acute lymphoblastic leukaemia (ALL), may be severely affected by the tropism of B19 to erythroid progenitor cells.

B19V is ubiquitous worldwide. A study based on the detection of B19V DNA in skin biopsies proposed that genotypes 1 and 2 in northern Europe were more prevalent in the past, but that genotype 2 was replaced by genotype 1 in the 1960s [24,25]. Conversely, B19V genotype 3 is found almost exclusively in Ghana, although it is also found in Brazil and France, and rarely in the USA [10,26–29]. It is possible that genotype 3 has emerged in Africa and, after a long evolution, has been introduced to Brazil and the USA, probably as a consequence of the large-scale influx of people of African origin through the slave trade in the eighteenth century.

Following an acute phase, the virus may establish a persistent infection that remains in human tissues for years or even a lifetime [22,25,30]. In patients with ALL, infection with B19V can cause chronic BM failure, usually manifested as anaemia, because of its tropism for human erythroid progenitors [31–33]. The impacts of B19V infection during maintenance chemotherapy can result in acute/chronic anaemia, pure red cell aplasia or pancytopenia [32]. Recently, it has been shown that patients with active B19 replication on maintenance therapy had longer periods with delayed or interrupted chemotherapy and required frequent blood transfusions [34]. Except for studies involving children with ALL, little to no information is available on the occurrence of B19V infection among adults with ALL or other types of leukaemia [34–38].

In the present study, we sought to determine the prevalence of B19V infection in all adult patients affected by leukaemia, including ALL, acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML), seen at our institution from 2007 to 2010. In addition, we conducted a molecular characterization of partial and larger fragments spanning the coding NS1/VP1/VP2 region of the isolated viruses with an aim to providing new genetic materials that could provide information on B19V evolution.

Materials and Methods

Patients

This study was retrospectively conducted on frozen BM samples consecutively collected at the time of diagnosis from 155 adult patients with AML, 78 patients with ALL, and 16 patients with CML. All patients were admitted to the Sao Paulo Clinical hospital from November 2007 to August 2010. According to the state health department bulletin there had been no epidemic outbreaks of erythema infectiosum in São Paulo during the study. At diagnosis, haematological parameters were determined for all the patients through standard methods. The diagnosis of ALL and AML was made through a morphological evaluation of BM biopsies for which immunophenotypic analysis for surface B-lymphocytic and T-lymphocytic markers was also performed. The CML diagnosis was confirmed by the presence of the *BCR-ABL* translocation by cytogenetic analysis, fluorescence *in situ* hybridization analysis, or molecular analysis as previously reported [39].

The characteristics of the 249 patients herein analysed are summarized in Table 1. Written consent was given by the patients for their information to be stored in the hospital database and used for research. This study was approved by the Institutional Ethical Research Board of the Hospital das Clínicas e da Faculdade de Medicina da Universidade de São Paulo.

Amplification and sequencing of B19V DNA

The genomic DNA used for the PCR analyses was extracted from BM samples with a QIAamp DNA Blood Mini Kit

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TABLE 1. Patient characteristics and findings

Characteristics	No. of samples		B19V genotypes	
	Total	B19V+	Genotype 1	Genotype 3
Total	249	40 (16%)	25 (10.04%)	15 (6.02%)
Sex				
Male	139	22 (15.83%)	13 (9.35%)	9 (6.47%)
Female	110	18 (16.36%)	12 (10.91%)	6 (5.45%)
Age, years				
18–29	99 (40%)	14 (15.56%)	14 (15.56%)	ND
30–39	63 (25%)	13 (20.63%)	8 (12.70%)	5 (7.94%)
40–49	27 (11%)	2 (7.41%)	ND	2 (7.41%)
>50	60 (24%)	11 (18.33%)	3 (5%)	8 (13.33%)
Leukaemia/lymphoma				
ALL	78 (31%)	12 (15.38%)	7 (8.97%)	5 (6.41%)
AML	155 (62%)	25 (16.13%)	17 (10.97%)	2 (12.5%)
CML	16 (7%)	3 (18.75%)	8 (5.16%)	1 (6.25%)

B19V, human parvovirus B19; ND, not detected; ALL, acute lymphoid leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia.

(Qiagen, Hilden, Germany), according to the manufacturer's instructions. To determine the prevalence of B19V infection, all extracted DNA was initially used as a template to amplify a 424-base-pair (bp) fragment as previously described [28]. Positive and negative controls (healthy donor polymorphonuclear leucocytes) were included in each assay. Samples positive for B19V were further confirmed by sequencing and phylogenetic analysis. For amplification and sequencing of the complete coding NS1/VP1/VP2 region (4413 nucleotides (nt)), we initially used the primers and protocols described by Toan *et al.* [11] (hereafter referred to as strategy 1), as these primers are able to uniquely amplify B19V genotype 1. We also designed a set of nested specific-genotype-3 primers to amplify two overlapping fragments that cover the entire coding NS1/VP1/VP2 region (4432 bp, nt 515 to 4947). A 2127-kilobase (kb) fragment corresponding to the 5′-region was amplified with the outer primers P1f and E3_n5r and the inner primers E3_n1f and P5r (see Table 2). The 3495-kb 3′-end fragment was amplified using the outer primers 103-OF and 103-OR and the inner primers 101-OF1 and 104-OR. The reaction mixtures for first-round PCR of both fragments contained 250–300 ng DNA template, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.5 μM of each primer and 2.5 U high-fidelity *Taq* platinum DNA polymerase (Invitrogen, Carlsbad, CA) in an $MgSO_4$ reaction buffer. After an initial denaturation of 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 55°C, 3 min at 72°C and a final extension at 72°°C for 5 min were performed. For the nested PCR, 5 μL of the first PCR was used, and the PCR mixture and thermal profile were the same as those described above. The β-globin gene targeting the TAL57 region was used to exclude PCR inhibitors and check for DNA integrity [40]. Each PCR included a known B19V DNA positive control and an interspersed no DNA template negative controls. The amplified fragments were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The purified products were directly sequenced on both strands using the PCR inner primers and, whenever necessary, additional internal specific primers (see Table 2) and the PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems/Perkin-Elmer, Foster City, CA) in an automated sequencer (ABI 3130, Applied Biosystems). After

TABLE 2. Primers used for polymerase chain reaction amplification and DNA sequencing of the coding NS1/VP1/VP2 regions of B19Vgenotype 3

Name	Orientation	Sequence 5′–3′	Position[a]
E3_N1F	Sense	CTGCTAACTAACAGGTATTTATACTAACT	515–547
E3_N1R	Antisense	TAATAATGTTCTTTACCAYCTTGTAACTGA	902–873
E3_N2F	Sense	GAAACTTAACTGTGYGTGTAGAAGG	842–866
E3_N2R	Antisense	GCAGTAGTCACAGTGGCAGCT	1318–1298
E3_N3F	Sense	TGGTAAATTGGCTATGTGAAAACAGAG	1217–1243
E3_N4F	Sense	GGCCAGCCAACCAGGGTAG	1729–1747
E3_N4R	Antisense	GGATTGCCTGTTTGTTGTGTGGAACAT	2373–2347
E3_N5F	Sense	TAGGGGTTGACTTTGTATGGGATGG	2315–2339
E3_N5R	Antisense	TACTGGTACAGATTTAGAACTTATTCAA	2686–2659
E3_N6F	Sense	ACCTGTCTGGGTTACAAAGCTTTG	2540–2563
E3_N6R	Antisense	GCTGGGCCTCCGCAGAATGC	3003–2984
E3_N7F	Sense	TACACAAGCCTGGGCAAGTTAG	2913–2934
E3_N7R	Antisense	GCTAGTGGGAAAGAGGCGAAAGTG	3472–3449
E3_N8F	Sense	AGCAACCCTACAAAAAGCATGTGG	3302–3325
E3_N8R	Antisense	GGTGAAGTAAACACACAGGGAGTT	3841–3818
E3_N9F	Sense	TAGCTCCAGAACTGCCCATTTGG	3759–3781
E3_N9R	Antisense	GTTACAAGGGCTAAACATGCACAC	4422–4399
E3_N10F	Sense	CTGGGACACTGATAAATATGTAACAGG	4267–4293
E3_N10R	Antisense	GGCAACCATCTGTAAAGGATACAG	5028–5005
103-OF	Sense	TAGTACTTTTTTGTTACATTCAGACTTTGAG	1431–1461
104-OR	Antisense	GGATATGAAAAGCCTGAAGAATTGTGGACT	4942–4913
103-OF	Antisense	GCCTGAAGAATTGTGGACTGCCAA	4947–4924
103-OF	Sense	TAGTACTTTTTTGTTACATTCAGACTTTGAG	1431–1461
104-OR	Antisense	GGATATGAAAAGCCTGAAGAATTGTGGACT	4942–4913
103-OF	Antisense	GCCTGAAGAATTGTGGACTGCCAA	4947–4924
P1f[b]	Sense	TGTGAGCTAACTAACAGGTATT	570–591
P5r[b]	Antisense	ATTCCACAAATTGCTGATACAC	2677–2699

[a]Nucleotide numbering according to position based on the sequence of B19 isolate J35 (GenBank accession no. AY386330).
[b]Toan *et al.*, 2006 [11].

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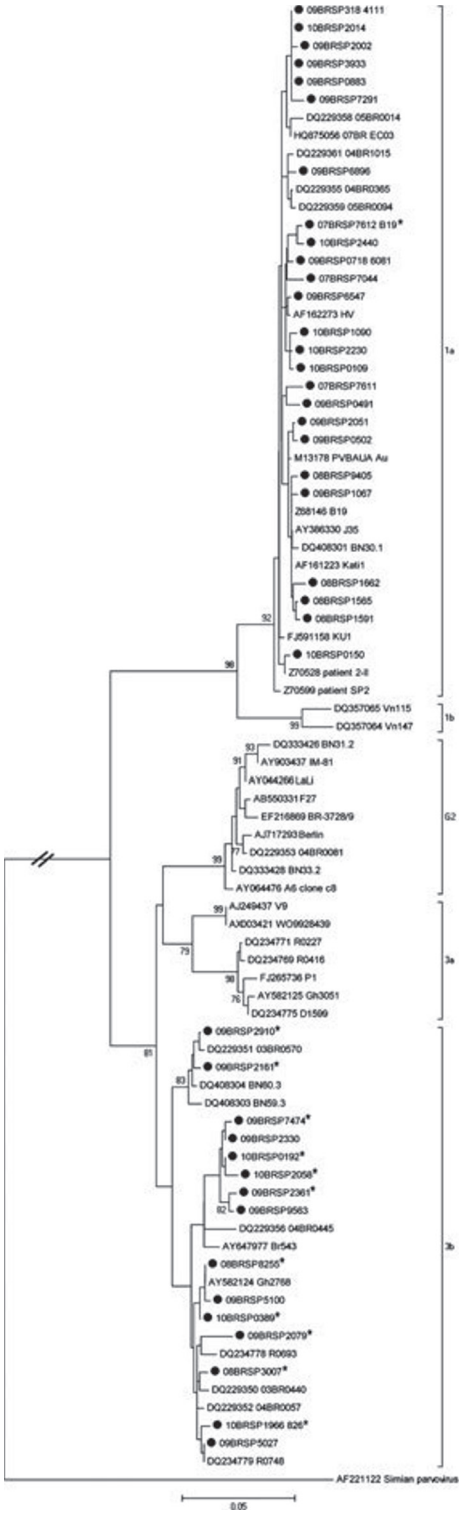
excluding the primer regions, the fragments for each amplicon were assembled into contiguous sequences and edited with SEQUENCHER 4.7 (Gene Code Corp., Ann Arbor, MI) using the default parameters.



Phylogenetic tree analyses and genotype determination

The B19V genotypes were determined by phylogenetic tree analysis, including all the B19V complete genomes of prototype strains (as of October 2011) from GenBank. Viral sequences were aligned using the CLUSTAL X program [41] Software alignment was followed by manual editing in the BIOEDIT SEQUENCE ALIGNMENT EDITOR program [42] to maximize similarity while maintaining consistency in the reading frames. The genetic distances were calculated by the maximum composite likelihood method, and phylogenetic trees were constructed using the neighbour-joining [43] method as implemented in MEGA version 5.0 software. The reliabilities of the branches were evaluated by bootstrap analysis with 1000 re-samplings. The sequences from which either partial or complete coding genomic regions were strongly positioned inside one of the genotype reference groups were compiled and their respective genotypes were assigned. The sequences suggestive of recombination by the above phylogenetic inferences that presented unclear clustering were subjected to recombination analysis. For this analysis, the bootscanning method [44] implemented in SIMPLOT v. 3.5.1 for Windows [43] was used with the following parameters: window size 250 bp, step size 20 bp, F84 model of evolution (maximum likelihood) as the model to estimate nucleotide substitution, transition/transversion ratio of 2.0, and a bootstrap of 100 trees. The significance threshold for the bootscan was set at 70%. The positions of crossover sites were defined based on the distribution of informative sites supporting the two incongruent topologies that maximized the chi-square value [45], a method implemented in SIMPLOT. Trees were displayed using MEGA version 5.0 software.

Statistical analysis

Differences between data groups were analysed with the Fisher's exact test or chi-square test with Yate's correction when appropriate. The two-tailed Mann–Whitney *U* test was employed for comparison of age differences between the groups as stratified by infecting B19V genotype. A p value <0.05 was considered significant. The data were analysed with STATA statistical software (StataCorp, release 5.0, 1997; Stata Corp., College Station, TX).

FIG. 1. Neighbour-joining inferred rooted phylogeny of a partial human B19V non-structural protein 1(NS1) sequence. The black circle denotes variants detected in patients surveyed in this study. The star symbol indicates patients dual-infected with genotypes 1a and 3b, as later determined by strategies 1 and 2. Bootstrap replication frequencies $\geq 70\%$ are indicated above nodes. Genotypes and subgenotypes are indicated at the corresponding nodes. The tree was rooted on Rhesus macaque parvovirus (GenBank AF221122). Branch lengths are drawn to scale.

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TABLE 3. The NS1/VP1/VP2 genotyping results of the NS1-positive samples as determined by strategy 1

Sample ID	Sequence fragment										Genotype
	$P1_{(580-950)}$	$P2_{(902-1367)}$	$P3_{(1322-1855)}$	$P4_{(1810-2367)}$	$P5_{(2356-2691)}$	$P6_{(2596-3037)}$	$P7_{(2982-3475)}$	$P8_{(3427-3898)}$	$P9_{(3851-4431)}$	$P10_{(4385-5035)}$	
09BRSP0491	+	+	+	+	+	+	+	+	+	+	Ia
08BRSP9405	+	+	+	+	+	+	+	+	+	+	Ia
07BRSP7044	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP2051	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP6896	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP6547	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP6081	+	+	+	+	+	+	+	+	+	+	Ia
08BRSP4111	+	+	+	+	+	+	+	+	+	+	Ia
10BRSP2230	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP2002	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP0883	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP1067	+	+	+	+	+	+	+	+	+	+	Ia
10BRSP2440	+	+	+	+	+	+	+	+	+	+	Ia
10BRSP2014	+	+	+	+	+	+	+	+	+	+	Ia
10BRSP0109	+	+	+	+	+	+	+	+	+	+	Ia
10BRSP0150	+	+	+	+	+	+	+	+	+	+	Ia
07BRSP7611	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP7291	+	+	+	+	+	+	+	+	+	+	Ia
09BRSP3933	+	+	+	+	+	+	+	+	+	+	Ia
10BRSP0826*	+	+	+	+	+	+	+	+	+	−	Ia*
07BRSP7612§	−	+	+	+	+	+	+	+	+	+	Ia/3b*
10BRSP1090	−	+	+	+	+	+	+	+	+	+	Ia
09BRSP0502	−	+	+	+	+	+	+	+	+	−	Ia
08BRSP3007*	−	+	+	+	+	+	+	+	+	−	Ia*
10BRSP0389*	−	−	+	+	+	+	+	+	+	+	Ia*
09BRSP2361*	−	−	−	+	+	+	+	+	+	+	Ia*
08BRSP1565	−	+	+	+	+	−	−	−	−	−	Ia
09BRSP2161*	−	+	+	+	+	−	−	−	−	−	Ia*
10BRSP2058*	−	+	+	+	+	−	−	−	−	−	Ia*
09BRSP2910*	−	+	+	+	+	−	−	−	−	−	Ia*
09BRSP0318	−	+	+	+	+	−	−	−	−	−	Ia
09BRSP2079*	−	+	+	+	+	−	−	−	−	−	Ia*
10BRSP0192*	−	−	−	−	−	+	+	+	+	−	Ia*
08BRSP1591	−	−	−	−	−	−	+	−	+	−	Ia
08BRSP1662	−	−	−	−	−	−	+	+	+	−	Ia
09BRSP7474*	−	−	−	−	−	−	+	+	+	−	Ia*
08BRSP8255*	−	−	−	−	−	−	+	+	−	−	Ia*

NS1/VP1/VP2, non-structural protein 1/viral capsid proteins 1 and 2
Nucleotide numbering according position based on the sequence of B19 isolate J35 (GenBank accession no. AY386330).
*Patients were found to be co-infected with genotype 3b as determined by strategy 2 (see Table 4). Partial sequences subjected to phylogenetic analysis (see Fig. 2) are indicated by shaded boxes.
§The patient was found to be coinfected with intergenotypic recombinant (1a/3b) and an NS1 non-recombinant genotype 1a.

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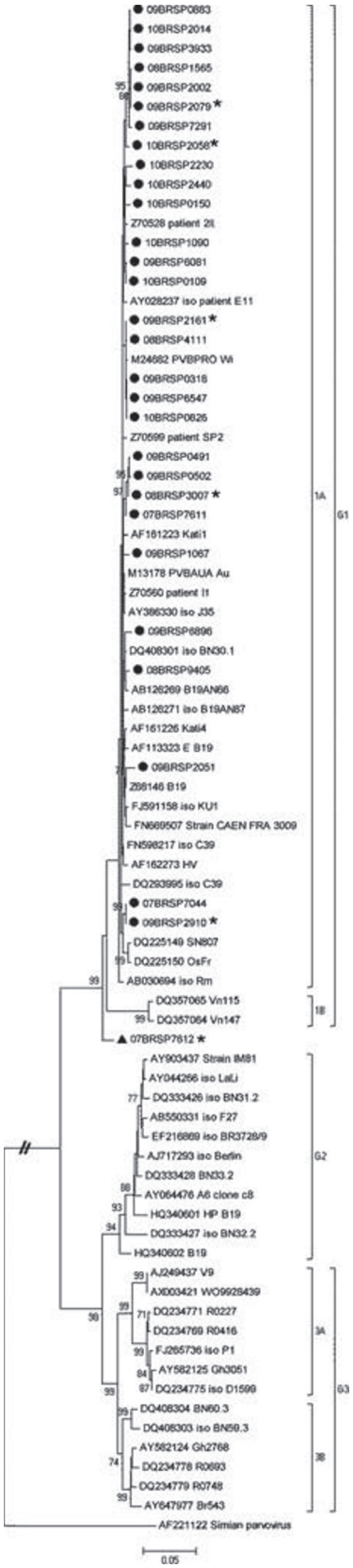

GenBank accession numbers

All nucleotide sequences were reported to the GenBank database (GenBank accession no. KC013302-KC013351).

Results

Amplification of the partial B19V NS1 genome was observed in 40 of 249 BM samples (16%) from patients with a confirmed diagnosis of leukaemia. Of these patients, B19V positivity was detected in 12 of 78 (15.4%) ALL patients, 25 of 155 (16.3%) AML patients, and 3 of 16 (18.7%) CML patients. No statistically significant differences were observed among the considered groups. Partial sequences of the NS1 gene were successfully obtained from all B19V-positive samples. Phylogenetic analysis of these sequences, together with 43 corresponding region sequences of selected B19V strains available in GenBank, revealed the presence of genotype 1a (62.5%) and genotype 3b (37.5%) viruses (Fig. 1). No sample was positive for the B19V genotype 2. Among the 78 ALL samples, seven isolates clustered with genotype 1a and five clustered with genotype 3b prototype strain sequences. Among the 155 AML specimens, 17 isolates were characterized as genotype 1a and eight as genotype 3b viruses. In 16 CML patients, one sample was characterized as genotype 1a and two as genotype 3b viruses. A pattern of sequence segregation was supported by >80% bootstrap values in all analyses, as indicated at the respective branches (Fig. 1). Among the partial NS1 genomic regions examined, the isolates closely related to the reference genotype 1 sequences had inter-strain genetic distances (*d*) of 8%, with a range of 0.3–16.7%. Within genotype 1a, the viral sequences were more closely related to each other (d = 0.9%); diversity between isolates ranged only from 0.3% to 2.4%. In contrast, the isolates closely related to the reference genotype 3 sequences had an inter-strain *d* of 8.2%, with a range of 0.3–17.5%. Within genotype 3b, virus sequences were less distantly related to each other (d =

FIG. 2. Neighbour-joining inferred rooted phylogeny of human B19V non-structural protein 1/viral capsid proteins 1 and 2 (NS1/VP1/VP2) sequence. The black circle denotes variants detected in patients with partial human B19V NS1 sequences. The star symbol indicates patients dually infected with genotype 3b, as later confirmed by strategy 2. The black triangle symbol indicates an isolate forming a unique branch in genotype 1 separate from 1a and 1b, which appeared in further analysis as a 1a/3b recombinant (see Fig. 3). Bootstrap replication frequencies $\geq$70% are indicated above nodes. Genotypes and subgenotypes are indicated at the corresponding nodes. The tree was rooted on Rhesus macaque parvovirus (GenBank AF221122). Branch lengths are drawn to scale.

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2.3%); diversity between isolates ranged only from 0.3% to 4.7%. The phylogenetic analysis of B19V sequences obtained in this study indicated that they did not form a single monophyletic cluster but instead belonged to genotype 1a and 3b in various subclusters, most of which had no significant bootstrap support.

To confirm the consistency of the genotyping, allow a more advanced phylogenetic analysis and provide a set of reference sequences for each viral genotype, the 40 samples with NS1 sequences were subjected to amplification and sequencing of the complete coding NS1/VP1/VP2 regions of B19V using strategy 1. Of these, 20 sequences were obtained for all ten overlapping fragments that covered the complete coding regions of the 40 patients. Partial sequences were obtained from at least one fragment derived from 16 samples as shown in Table 3. In the phylogenetic tree of the partial sequences shaded in Table 3, isolate 07BRSP7612 forms a unique branch in genotype 1, suggesting a new subgroup (Fig. 2). However, further analysis using SimPlot and bootscanning of the amplified fragment indicated a single recombinant event between genotype 1a (between nt 949 to 1879) and genotype 3b (between nt 1886 to 2160) (Fig. 3a). To further confirm this result, phylogenetic trees were generated for the nucleotide sequence regions on either side of the breakpoint. This analysis corroborated the results from the bootscan and so provides unambiguous evidence for recombination events supported by high bootstrap values (Fig. 3b). Crucially, the breakpoint was detected in a position far from the regions containing sequences identical or complementary to the sequences of oligonucleotide primers used for PCR or sequencing, demonstrating that this breakpoint is indeed a *bona fide* recombinant rather than a laboratory artefact. Next, we aimed to investigate the phylogenetic correlation of the genotype 3b fragment involved in the recombinant event as defined by bootscan and the non-recombinant genotype 3b fragment from the same patient as defined by strategy 2 (Table 4). The result of the neighbour-joining tree depicted in Fig. 4 revealed both sequences clustered on a genotype 3 branch and further into one separate sub-branch supported by a high bootstrap value (86%). Moreover, the alignment of the

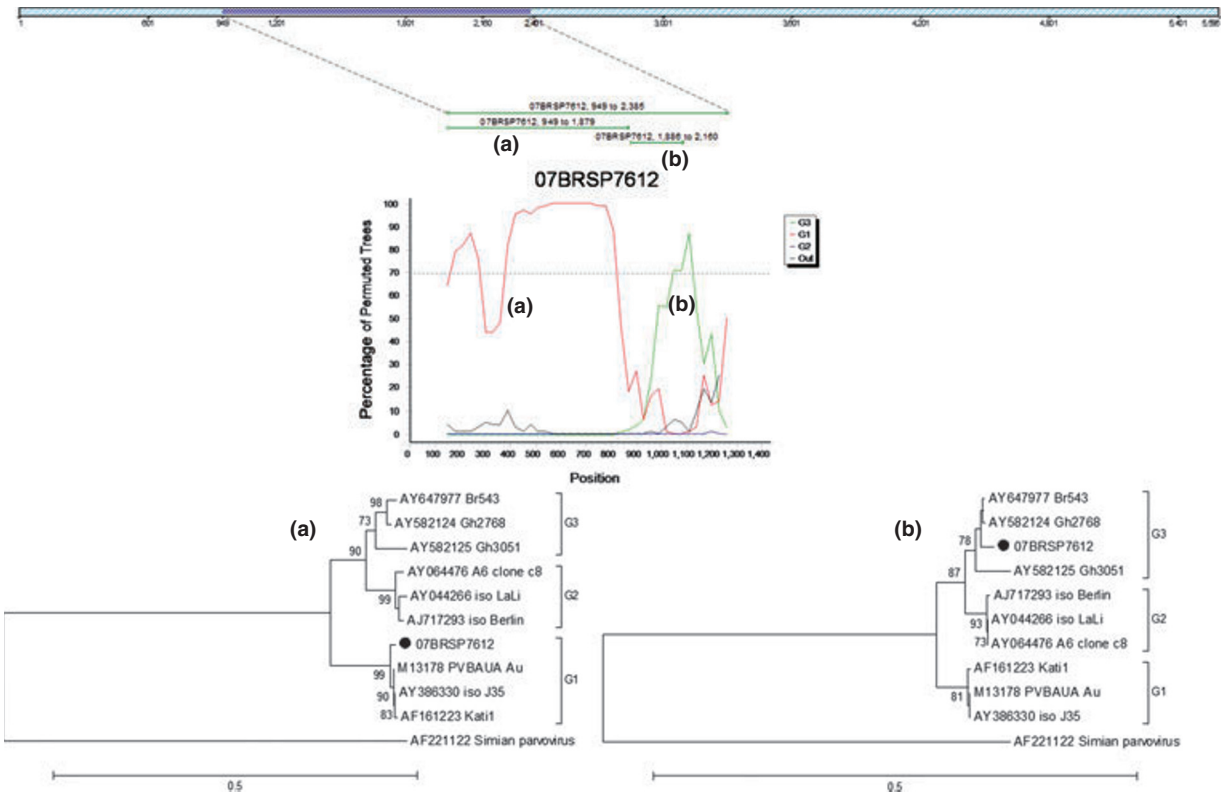

FIG. 3. Exploratory tree analysis based on fragments between breakpoints as indicated by a bootscan plot of isolate 07BRSP7612. The neighbour-joining tree was established from the alignments of the non-structural protein 1 (NS1) fragment spanning nucleotide positions 949–1874 (a) and positions 1886–2160 (B19 isolate J35 GenBank accession no. AY386330) (b) for isolate 07BRSP7612 (indicated by black circle) and other B19V reference genotypes. Bootstrap replication frequencies $\geq$70% are indicated above nodes. Genotypes and subgenotypes are indicated at the corresponding nodes. The tree was rooted on Rhesus macaque parvovirus (GenBank AF221122). Branch lengths are drawn to scale.

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TABLE 4. The NS1/VP1/VP2 genotyping results of the NS1-positive samples as determined by strategy 2

Sample ID	Sequence fragment									Genotype
	P1$_{(515-902)}$	P2$_{(842-1318)}$	P3$_{(1217-1817)}$	P4$_{(1729-2373)}$	P5$_{(2315-2686)}$	P6$_{(2540-3003)}$	P7$_{(2913-3472)}$	P8$_{(3302-3841)}$	P9$_{(3759-4422)}$	P10$_{(4267-5028)}$	
09BRSP9563	+	+	+	+	+	+	+	+	+	+	3b
09BRSP2330	+	+	+	+	+	+	+	+	+	+	3b
09BRSP5027	+	+	+	+	+	+	+	+	+	+	3b
09BRSP2079*	+	+	+	+	+	+	+	+	+	+	3b*
10BRSP1966*	+	+	+	+	+	+	+	+	+	+	3b*
10BRSP0389*	+	+	+	+	+	+	+	+	+	+	3b*
10BRSP0192*	+	+	+	+	+	+	+	+	+	+	3b*
08BRSP8255*	+	+	+	+	+	+	+	+	+	−	3b*
09BRSP7474*	+	+	+	+	+	+	+	+	+	−	3b*
10BRSP2161*	+	+	+	+	+	+	+	+	+	−	3b*
09BRSP5100	−	−	+	+	+	+	+	+	+	+	3b
09BRSP2361*	−	−	+	+	+	+	+	+	+	−	3b*
07BRSP7612*	−	−	+	−	−	−	−	−	−	−	3b*
09BRSP2910*	−	−	+	−	−	−	−	−	−	−	3b*
08BRSP3007*	−	−	+	−	−	−	−	−	−	−	3b*
10BRSP2058*	−	−	+	−	−	−	−	−	−	−	3b*

NS1/VP1/VP2, non-structural protein 1/viral capsid proteins 1 and 2
Nucleotide numbering according position based on the sequence of B19 isolate J35 (GenBank accession no. AY386330).
*Patients were found to be co-infected with genotype 3b as determined by strategy 2 (see Table 4).

sequenced fragments did slightly differ from each other in the manner expected for related genotype 3 viruses that have diverged through independent evolution. Based on this finding, it is possible that the recombination event that produced the 1a/3b isolate took place in patient 07BRSP7612, who was found to also be co-infected with non-recombinant genotype 3b strains. In contrast to recombination analysis, visual inspection of a tree constructed from the samples with complete coding NS1/VP1/VP2 regions (Tables 3 and 4) revealed that sequence 07BRSP7612 grouping with B19V genotype 1a with high statistical support (data not shown). This observation was not surprising, given the short sequence of the putative recombinant region (Fig. 5).

Surprisingly, when analysed with other B19V reference sequences, all strains obtained by strategy 1 clustered significantly with B19V genotype 1a (Fig. 2). The conflicting phylogenetic data, such as the data obtained for the partial NS1 and the complete coding NS1/VP1/VP2 sequences in samples marked with a star symbol in Tables 3 and 4 and Fig. 2, were indicative of the presence of a mixed infection of subgenotype 1a and and subgenotype 3b. These results also raised doubts about the specificity of the primers used in strategy 1 to amplify genotype 2 and 3. As a consequence, the BLAST search of each primer indicated high bias in favour of genotype 1 and 2 amplification. These findings prompted us to search for an alternative approach to enable construction of a phylogenetic tree of B19V genotype 3 from longer fragments (hereafter referred to as strategy 2). For that purpose, we used strategy 2 to amplify and sequence the 40 samples positive for NS1 sequences as mentioned in the Materials and Methods. The strategy 2 amplification results are summarized in Table 4. With the exception of sample 07BRSP7612, subsequent phylogenetic analysis of the amplified products indicated identical genotyping 3b results to those obtained by analysis of the partial NS1 fragment. The phylogenetic analysis of the sequences obtained by strategies 1 and 2 from sample 07BRSP7612 indicated that this patient had been doubly infected with genotype 1a/3b recombinant and genotype 3 viruses. In all partial and long-fragment analyses, no identical virus sequences were found among the 40 B19V-infected patients, confirming the absence of PCR contamination. Indeed, we could not see any signal of mixed bases on the initial analysis of the NS1 partial sequences from the dually infected samples (as defined later by the genotype-specific primers used for strategies 1 and 2). Although quantification of viral load was not performed in our subjects, it is possible that mixtures of different genotypes were present in the same sample and the identification of both genotypes may be compromised if one of the other viral variants is present at a lower load.

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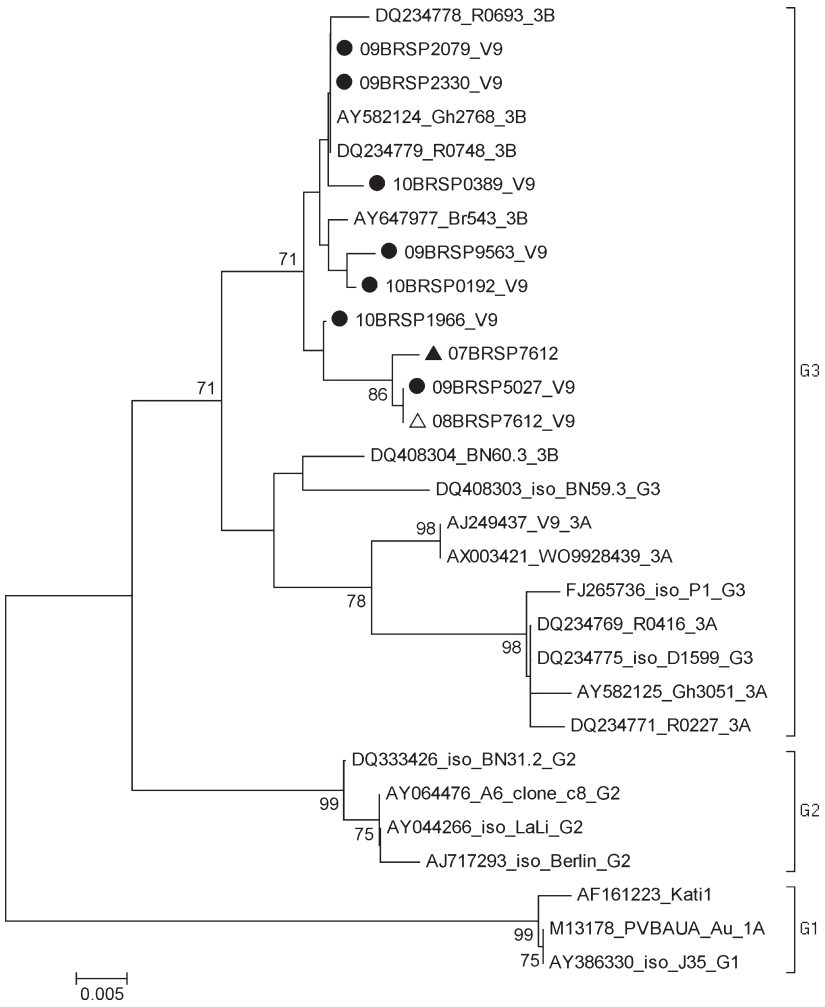

FIG. 4. Neighbour-joining inferred rooted phylogeny of the genotype 3b fragment involved in the recombinant event as defined by bootscan (274 base pairs, nucleotides 1886–2160 of B19 isolate J35 GenBank accession no. AY386330) and the non-recombinant genotype 3b fragment as defined by strategy 3 from the 07BRSP7612 isolate (indicated by black circle). Bootstrap replication frequencies $\geq$65% are indicated above nodes. Genotypes and subgenotypes are indicated at the corresponding nodes. The tree was rooted on simian parvovirus (GenBank accession no AF221122). Branch lengths are drawn to scale.

The detection of both viruses in a single clinical sample strongly suggests that the presence of the two variants was the result of either co-infection or superinfection. However, only the frequency of dual infection was noted in this study because we do not know whether co-infection or superinfection originally occurred.

In our samples, subjects infected with non-recombinant genotype 3b viruses were older than patients infected with genotype 1 viruses. However, the differences between the two groups did not reach statistical significance because sample sizes were very small for the patients only infected with genotype 3b (n = 4; mean age, 31.6 years versus 46.7 years, respectively; p 0.051, Mann–Whitney U test). For this reason, patients mono-infected with genotype 3 and patients co-infected with genotypes 1a, 3b and 1a/3b recombinant were grouped together and compared with patients infected with non-recombinant genotype 1 in subsequent analyses. This analysis revealed a marked difference between the groups (mean age, 62.5 years versus 47.2 years, respectively; p <0.01, Mann–Whitney U test) (Table 5).

Discussion

In this study, we aimed to investigate the putative occurrence of B19V in adults with different types of leukaemia. The 16% prevalence of B19V positivity in this group of patients is in the range of 5.7–68.8% in children and adults with haematological disorders, including leukaemia, that has been reported by our group and others [17,21,23,28,34,46,47]. The divergence in prevalence rates may reflect geographic differences in the spread of the virus in diverse parts of the world, methodological differences in diagnostic procedures, differences in sample size, or differences in the degree of immunosuppression associated with the duration and dose intensities of patient chemotherapies. Using phylogenetic analysis, we demonstrated that at least two B19V genotypes, 1a and 3b, are currently present in patients with leukaemia. In agreement with previous studies, our results indicated significantly well-defined subgenotypes within genotypes 1 and 3 [5,48,49]. Moreover, we did not find any sample positive for genotype 2.

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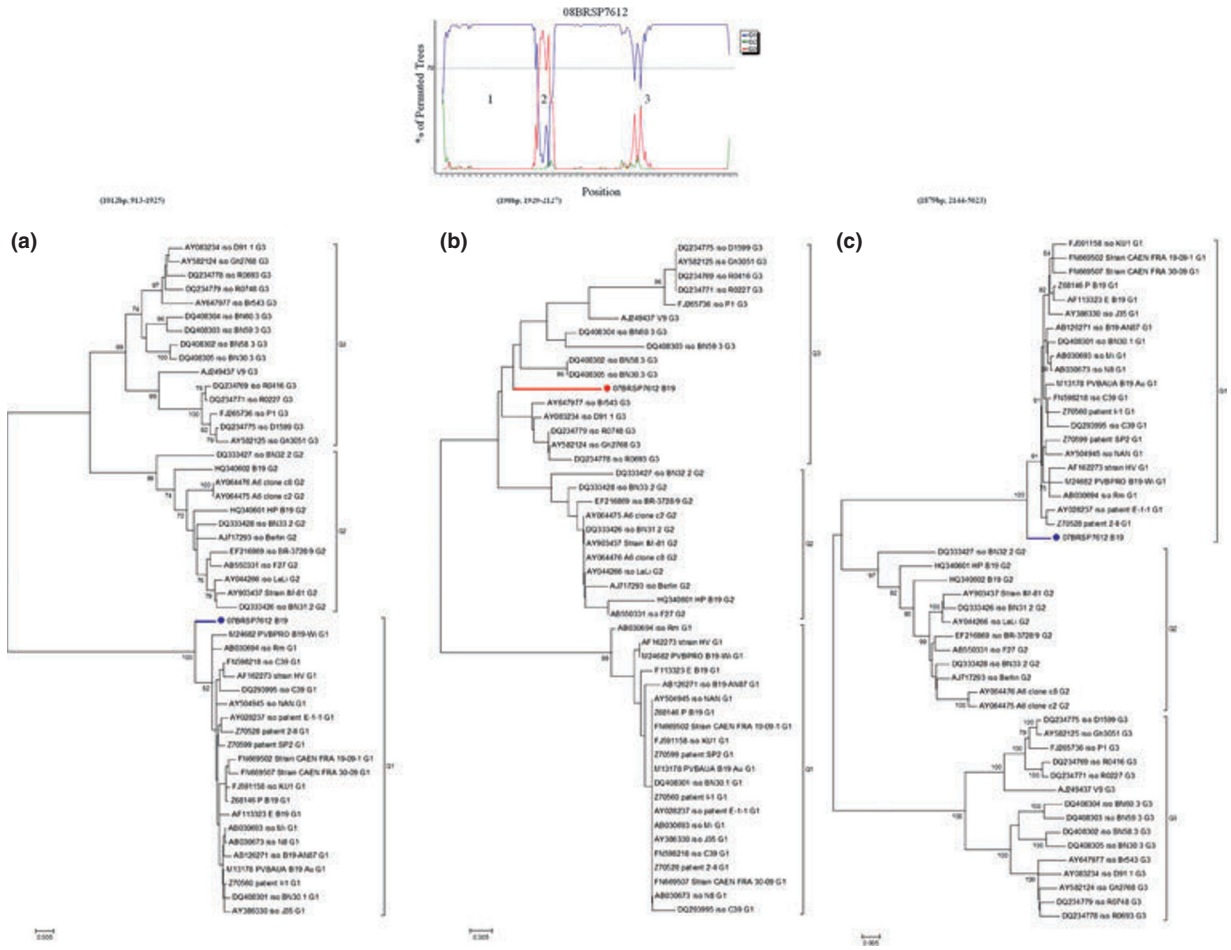

FIG. 5. Exploratory tree analysis based on fragments between breakpoints as indicated by a bootscan plot of isolate 07BRSP7612 complete coding non-structural protein 1/viral capsid proteins 1 and 2 (NS1/VP1/VP2) sequence. The neighbour-joining tree was established from the alignments spanning nucleotide positions 913–1925 (a), 1929–2127 (b), and positions 2144–5023 (c) (B19 isolate J35 GenBank accession no. AY386330). Isolate 07BRSP7612 is indicated by circle. Bootstrap replication frequencies $\geq$70% are indicated above nodes. Genotypes are indicated at the corresponding nodes. Branch lengths are drawn to scale.

As the partial NS1 PCR assay used was equally sensitive to B19V variant genomes, the lack of genotype 2 most likely indicates a real absence of this genotype from the subjects examined in the present study. Alternatively, it is possible that we may have failed to amplify genotype 2 in some patients if they had B19V levels below the detection limit of our assay.

The detection of genotypes 1 and 3 in Brazilian patient samples was expected. The majority of B19V Brazilian isolates described have been affiliated with these genotypes [23,28,48,49]. The results of phylogenetic analysis indicated that genotype 3b sequences from Brazil did not group as a specific, closely related cluster as was observed with the isolates from genotype 1a. Moreover, the genotype 1a isolates were more closely related to each other than the genotype 3b isolates. These observations may suggest that B19V genotype 3b has been present in Brazil for a very long time and that genotype 1a isolates were introduced relatively recently. Although speculative at this point, this observation should be amenable to robust phylogenetic testing analysis like a Bayesian coalescent approach on more sequences with time information to understand the origin and evolution of B19V genotypes in Brazil.

Our results also demonstrated that patients infected only with non-recombinant genotype 1a were on average younger than patients who were mono-infected or co-infected with genotype 3b. These results are in accordance with data collected from central and northern Europe, suggesting that the circulation of genotype 2 viruses ceased in the late 1960s and that genotype 1 is now the most prevalent in those areas [24,25]. The fact that the majority of patients infected with genotype 1a were younger on average suggests that different B19V genotypes do not induce distinct immunological responses.

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TABLE 5. Summary of B19V-infected patients surveyed

Sample ID	Age[b]	Gender	Leukaemia	B19V genotype
07BRSP7044	35	F	AML	1a (C)
07BRSP7611	30	M	AML	1a (C)
07BRSP7612[c]	22	M	AML	1a (C)/3b (P)
08BRSP1565	68	M	AML	1a (P)
08BRSP1591	73	M	AML	1a (P)
08BRSP1662	58	F	AML	1a (P)
08BRSP3007[a]	60	F	AML	1a (C)/3b (P)
08BRSP4111	32	M	ALL	1a (C)
08BRSP8255[a]	76	M	AML	1a (P)/3b (C)
08BRSP9405	26	F	AML	1a (C)
09BRSP0491	25	F	AML	1a (C)
09BRSP0502	18	M	AML	1a (C)
09BRSP0883	18	M	AML	1a (C)
09BRSP1067	36	M	CML	1a (C)
09BRSP2002	18	F	ALL	1a (C)
09BRSP2051	34	F	AML	1a (C)
09BRSP2079[a]	60	M	AML	1a (P)/3b (C)
09BRSP2330	54	F	ALL	3b (C)
09BRSP2361[a]	36	F	ALL	1a (P)/3b (P)
09BRSP2910[a]	45	M	AML	1a (P)/3b (P)
09BRSP3933	23	M	ALL	1a (C)
09BRSP5027	30	F	ALL	3b (P)
09BRSP5100	61	F	AML	3b (P)
09BRSP6081	33	F	ALL	1a (C)
09BRSP6547	28	M	AML	1a (C)
09BRSP6896	28	M	AML	1a (C)
09BRSP7291	23	M	ALL	1a (C)
09BRSP7474[a]	38	M	AML	1a (P)/3b (C)
09BRSP9563	42	M	CML	3b (C)
10BRSP0109	37	F	AML	1a (C)
10BRSP0150	20	F	ALL	1a (C)
10BRSP0192[a]	53	M	ALL	1a (P)/3b (C)
10BRSP0389[a]	59	F	ALL	1a (P)/3b (C)
10BRSP1090	18	F	AML	1a (C)
10BRSP1966[a]	33	M	AML	1a (C)/3b (C)
10BRSP2014	18	F	AML	1a (C)
10BRSP2058[a]	39	M	CML	1a (P)/3b (P)
10BRSP2161[a]	66	M	AML	1a (P)/3b (C)
10BRSP2230	22	F	AML	1a (C)
10BRSP2440	38	M	ALL	1a (C)

F, female; M, male; P, partial genome; C, complete coding NS1/VP1/VP2 region (non-structural protein 1/viral capsid proteins 1 and 2); B19V, human parvovirus B19; ND, not detected; ALL, acute lymphoid leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia.
aEvidence of genotype 1a and 3b dual infection;
bDifference in age between infection involving genotypes 3, and genotype 1, $p < 0.01$ (Mann–Whitney *U* test).
cEvidence of recombination event.

The observation that patients may be simultaneously infected with different B19V genotypes is of considerable interest. Schneider *et al.* [50] found that 4 of 59 study participants presented the simultaneous persistence of two genotypes. In another study, Corcioli *et al.* [51] noted mixed infections by genotype 1 and 2 in one bioptic sample and also observed DNA of both genotypes in two other patients who had genotype 1 in their serum samples and genotype 2 in their tissue samples. In the present study, there was a relatively high rate of dual infections involving genotypes 1a and 3b, suggesting that this event is far more common in Brazil where both genotypes co-circulate. Furthermore, it is likely that B19V co-infection/superinfection occurred repeatedly in patients with leukaemia, given that 12% of our subjects were co-infected with genotypes 1a and 3b. The fact that our patients had a relatively high rate of dual infection goes against the hypothesis that an initial B19V infection produces some degree of protection against second infecting genotypes. If we assume that superinfection occurs, then it is conceivable that these patients may have been less able to prevent infection with a second strain than they would have been if they had had sufficiently broad protection at the time of primary infection, as has been speculated by Schneider *et al.* [50]. The impact of repeated exposure to B19V may be the weakening or exhaustion of the virus-specific immune response, making an individual more likely to be re-infected. Another possibility is that these patients were re-infected before their primary adaptive response had sufficiently matured. Alternatively, the subjects may have been concomitantly infected with two genotypes at the same time, but this is less likely. It is evident that new research with longitudinally collected samples is needed to investigate the cellular immune responses against the primary viral variant and a subsequent re-infection variant to support or refute the hypothesis of cross-genotype immunity and protection against B19V persistent infection.

On the other hand, frequent detection of coexisting B19V distinct genotypes might invariably lead to recombination [6, 52]. The latter was indeed observed in one sample infected with genotype 1a/3b recombinant and genotype 3 viruses. To our knowledge, the present report is the first to describe the occurrence of inter-genotype B19V recombinant in a single host. It is possible that recombination of intra- and inter-B19V genotypes occurs in countries where different viral genotypes co-circulate, but the current techniques for assigning consensus sequences will greatly hinder their detection except in cases where the genotypes represent the dominant viral population or more studies based on conventional clonal or high-throughput sequencing are undertaken.

Possible intergenotypic recombination mechanisms remain unclear. One possibility is that the positive and negative strands of different B19V single-stranded variants anneal during replication, as has been described with adeno-associated virus recombinants [53]. The fact that we do not yet know whether recombination poses a real threat justifies a more thorough investigation into the nature of this phenomenon in B19V viruses.

Our study has several limitations, particularly its retrospective design, lack of a control group and the absence of data regarding the clinical symptoms associated with B19V infection. In addition, the lack of a quantified B19V viral load and serological work-up limit the diagnostic accuracy of our analysis to qualitative B19V DNA PCR. As the mere presence of B19V in BM does not predict a pathogenic role, the best technical method to prove a pathogenic role for B19V may involve quantitative PCR, serological testing, or both. In spite of these caveats, our data add to the knowledge of the prevalence of B19V single or mixed infections caused by genotype 1 and 3 viruses in patients with different type of

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leukaemia and provide information from a country where such a phenomenon is rarely documented.

Overall, the data presented demonstrates a relatively high prevalence of B19V mono-infection and co-infection in patients with leukaemia and provides, for the first time, evidence of intergenotype recombination in a single patient. Such recombination may contribute to the genetic diversity of B19V and may also be a source of emergence for new viral strains. Detection of B19V DNA in patients with leukaemia as described in the present study does not prove causality. The virus might merely be an innocent passenger that was detected by chance or might become clinically relevant in this population as a result of treatment, as patients may become vulnerable to reactivation of persistent virus or re-infection following BM transplantation and the introduction of immunosuppressants. Therefore, further investigation of a large population of leukaemia patients using well-matched control groups and sequential samples will be required to elucidate the possible pathogenic impact of B19V infections.

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Transparency Declaration

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