Sequence Variability of Human Erythroviruses Present in Bone Marrow of Brazilian Patients with Various Parvovirus B19-Related Hematological Symptoms

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The presence of erythrovirus infections was investigated by PCR with bone marrow samples of patients with various parvovirus B19-related hematological symptoms. Erythrovirus DNA was found in 17.3% (12/69) of patients. Phylogenetic analysis revealed that five strains cluster with genotype 1, one clusters with genotype 2, and six cluster with genotype 3. Our study is the first to document the presence of the three erythrovirus genotypes in Brazil.

The human parvovirus B19 belongs to the genus *Erythrovirus* and is the only member of the family *Parvoviridae* to cause a wide range of human diseases, including erythema infectiosum, arthropathy, transient aplastic crisis, persistent anemia, and hydrops fetalis (13). Evidence of persistent infection without clinical manifestations has been reported to occur in immunocompetent individuals by detection of viral DNA in bone marrow and synovia years after infection (12). Ongoing virus replication in immunocompromised patients usually results in severe chronic anemia (4).

The virus is nonenveloped, and its genome consists of a linear, single-stranded DNA molecule of approximately 5,600 nucleotides (nt) with terminal palindromic inverted sequences of 383 nt at both ends (11). It has two main open reading frames (ORFs) encoding three functional proteins. The first ORF codes for the nonstructural protein NS1, while the second ORF expresses two structural proteins known as viral protein 1 (VP1) and viral protein 2 (VP2) (5).

Recent studies have shown many more genetic variations among erythrovirus variants than previously thought (7, 9, 10) and have suggested reclassification of these variants into three distinct genotypes, designated genotype 1 (B19-related viruses), genotype 2 (A6-related viruses), and genotype 3 (V9-related viruses) (10). While a significant amount of research has been conducted on genotype 1, little is known about the distribution of the other genotypes. Available evidence indicates some geographical restrictions: genotype 3 has been detected in samples from French patients (10) and Ghanaian blood donors but not among blood donors of Finnish, Danish, British, or African origin (2, 6, 8). However, there is no information regarding the molecular epidemiology and genetic variability of erythrovirus variants other than those of genotype 1 in Brazil. We therefore, studied the distribution of erythrovirus genotypes in Brazilian patients and explored their molecular characterization.

From December 2003 to March 2005 we received a total of 69 bone marrow samples from hospitalized patients with various parvovirus B19-related symptoms based on clinical grounds and hematological examinations. Samples were collected as part of the routine diagnosis of hematological disorders and sent to our laboratory at the request of the hematologist. Of the 69 subjects, 54 were adults (22 males and 32 females) and 15 were children below the age of 12 years (8 boys and 7 girls). Serologies for B19 virus-specific antibodies of these subjects were not available.

Genomic DNA was extracted from bone marrow samples using the Qiamp blood kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Because of the unreliability of the standard B19 virus PCR assay to detect genotypes 2 and 3, primers newly designed in-house were generated from the viral NS1 conserved regions of the three genotypes. PCR primers were designed by the Primer3 program (Whitehead Institute for Biological Research, http://www.genome.wi.mit.edu/) and were further analyzed for erythrovirus specificity using the Web-based BLAST program provided by the National Center for Biotechnology Information. The extracted DNA was used as a template to amplify a fragment of 424 bp (nt 1267 to 1691, corresponding to those of the prototype strain Pvbaua [GenBank accession no. M13178]) in a seminested-PCR fashion. Briefly, the outer primers 101-OF (5′-CATWCAGACTTTGAGCAGGTTA-3′ [nt 1267 to 1288]) and 101-OR (5′-TTATTGCCCAGTTTTCATAGTG-3′ [nt 1855 to 1876]) were used in the first-round amplification and yielded a product of 610 bp. The internal primers 102-NR (5′-GTAATGTCACCATTGCTGGTTAT-3′ [nt 1669 to 1691]) and 101-OF were used in the second-round amplification and yielded a 425-bp fragment. The PCR mixture was carried out in a 50-μl reaction volume containing 5 μl 10× Tris (pH 8.3; Invitrogen, Brazil), 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.2 mM concentrations of each of the deoxynucleoside triphosphates, and 1.5 μM units *Taq* polymerase (Invitrogen, Brazil). The amplification was performed in an Eppendorf gradient PCR cycler (Eppendorf Scientific) using an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 94°C for 45 seconds, 58°C for 45 seconds, and 72°C for 1 min, followed by 72°C for 10 min. A volume of 5 μl of the first-round reaction was amplified with

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TABLE 1. Characteristics of patients infected with different erythrovirus genotypes[a]

Sample	Yr	Age	Sex	Genotype	Clinical history and hematological manifestations
03BR0440	2003	34	F	3	Endometriosis, febrile pancytopenia, hemophagocytic syndrome
03BR0570	2003	60	F	3	Hepatic transplantation, colitis, pancytopenia
04BR0057	2004	63	F	3	Hepatic transplantation, anemia
04BR0081	2004	68	M	2	Renal transplantation, HHV6 positivity, pancytopenia
04BR0290	2004	59	M	3	Hepatic transplantation, diarrhea, pancytopenia
04BR0365	2004	39	M	1	Burkitt's lymphoma, AIDS, colitis, febrile pancytopenia
04BR0445	2004	61	F	3	T-cell leukemia, anemia, neutropenia
04BR0448	2004	55	M	3	Diabetes, febrile pancytopenia, hemophagocytic syndrome
04BR1015	2004	14	F	1	Myelodysplastic syndrome, BMT, rash, anemia thrombocytopenia
05BR0014[b]	2005	39	F	1	Skin rash, fever
05BR0094	2005	7	M	1	Adrenoleukodystrophy, BMT, HC, adenovirus positivity, pancytopenia
05BR0127	2005	31	M	1	AIDS, pure red cell aplasia

[a] HHV6, human herpesvirus 6; BMT, bone marrow transplantation; HC, hemorrhagic cystitis; F, female; M, male.
[b] Hematological manifestations was not available.

the inner-primer set for an additional 30 cycles, using the same conditions as in the first round. The PCR detection sensitivity was evaluated by serial dilutions of a viral quality control B19 DNA (VQC; Sanquin-CLB, Alkmaar, The Netherlands) standard containing a known quantity of viral DNA and mixed with erythrovirus-negative genomic DNA (polymorphonuclear cells), which were then amplified by seminested PCR. The assay allowed us to detect 250 geq/ml in a background of 2×10^5 human cells. The amplified products were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized under UV illumination.

Both complementary strands were directly sequenced with the internal primers, fluorescent-dye terminators, and *Taq* polymerase on an automated sequencer (ABI 3100; Applied Biosystems, Inc., Foster City, CA). A consensus sequence of both strands was formed by the Sequencher program (Gene Code Corp., Ann Arbor, Mich.).

Sequences were aligned with genotyped erythrovirus reference sequences from GenBank (genotype 1, B19-AN87 [AB126271], PVBAUAB19-Au [M13178], Kati4 [AF161226], B19-AN66 [AB126269], PVB19X560 [Z70560], J35 [AY386330], and Kati1 [AF161223]; genotype 2, Berlin [AJ717293], A6c8 [AY064476], A6c2 [AY064475], and LaLi [AY044266]; and genotype 3, Br543 [AY647977] and D91.1 [AY083234]) by the CLUSTAL X program (http://www-igbmc.u-strasbg.fr/BioInfo /ClustalX/) and analyzed by the PAUP version 4.0 beta10 win program written by D. L. Swofford (Sinauer Associates, Sunderland, MA) and MrBayes v3.1 (http://mrbayes.csit.fsu.edu/). The Modeltest software, v3.6 (http://darwin.uvigo.es/11), was used to test for a statistically justified model of DNA substitution that best fitted our data set. The evolutionary model Hasegawa-Kishino-Yano plus gamma was selected by the likelihood ratio test and used for further analysis. Bayesian tree searches were based on 1 million generations with four incrementally heated simultaneous Markov Chain Monte Carlo chains. Chains were started from random trees, which were sampled every 100th generation. Strains that clearly cluster inside one of the representative genotypes and showed statistical support of >70% were classified according to their reference group.

The presence of erythrovirus DNA was investigated in a total of 69 bone marrow samples from patients suspected of genotype 1 infection. Partial NS1 viral genomes were detected in 12 (17.3%) samples. The review of the medical records of these 12 patients showed that 6 had had a solid organ or bone marrow transplant. Infection with human immunodeficiency virus type 1 and human herpesvirus 6 was reported for two and one of these patients, respectively. Two other cases had evidence of hemophagocytic syndromes associated with pancytopenia. Other characteristics of these patients are presented in Table 1. Erythrovirus genotypes were determined from the 12 positive samples and assigned based on the portion of the NS1 gene sequenced, which, together with 14 published sequences from GenBank, provided a total of 26 sequences available for analysis. Several approaches to phylogenetic analysis, including maximum parsimony, distance, and Bayesian methods, were applied. All methods showed identical or nearly identical topologies. Results presented here used the Hasegawa-Kishino-Yano-plus-gamma model to calculate genetic distances and the Bayesian method to generate the tree (Fig. 1). The signal content of our data set was evaluated by the permutation tail probability test (1) and found to be well suited to robust phylogenetic reconstruction. All of the sequences segregated into one of the three erythrovirus genotypes, reproducing the same evolutionary relationship described previously (10) and indicating that the short fragments of the NS1 gene used in this study are adequate to allow genotype identification. As depicted in the figure, five sequences clustered with the genotype 1 reference group, showing nucleotide distances from 0 to 1.5%. One isolate (04BR0081) fell into the genotype 2 reference group in 100% of the posterior probability values. The last six isolates segregated with genotype 3 strains exhibiting nucleotide distances from 0.3 to 3.7%. Isolates were then grouped according to their genotype references and compared on the basis of their genetic distances. Genotype 1 strains differed from genotype 2 and 3 strains by nucleic acid distances of 14.7% and 13.2%, respectively. The interisolate distances of genotypes 2 and 3 were 5.8%. These results are almost consistent with the previous analysis of the complete NS1 region from all erythrovirus variants (10).

In this study, we report the detection of erythrovirus DNA in bone marrow samples from 17.3% (12/69) patients with symptoms suggestive of erythrovirus genotype 1 infection. Sequence analysis of a 424-bp fragment of viral DNA encoding a portion of the NS1 protein allowed the construction of phylogeny that
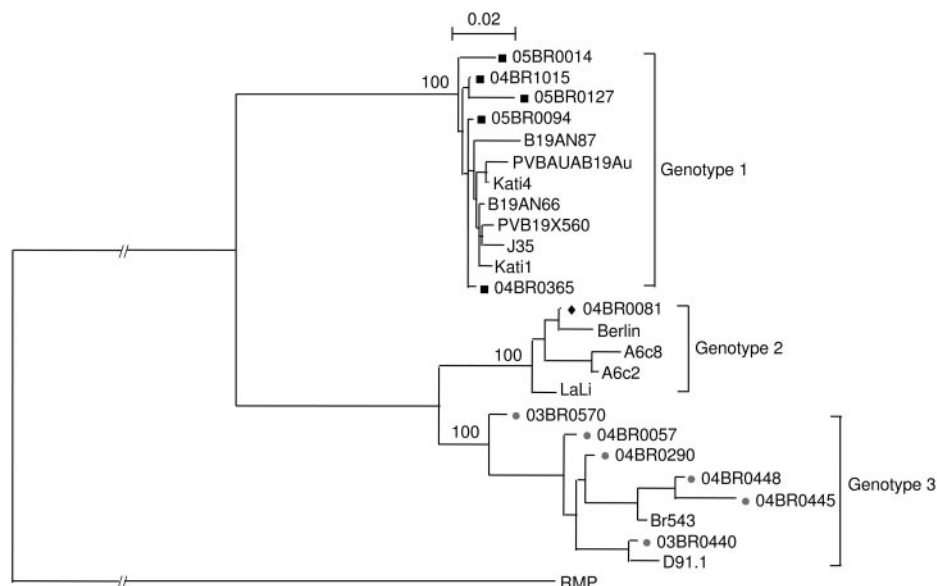
FIG. 1. Bayesian inferred rooted phylogeny of a partial human erythrovirus NS1 sequence, generated with MrBayes v3.7. Branch confidence intervals are given as posterior probability values. Black squares denote subjects classified as genotype 1, a black diamond denotes a subject classified as genotype 2, and grey circles denote subjects classified as genotype 3. The tree was rooted on Rhesus macaque parvovirus (RMP AF221122). Branch lengths are drawn to scale.

were equivalent to those obtained using the complete viral sequence. In the current genotyping system, the majority of isolates were clustered with genotype 1 (5/12) and genotype 3 (6/12) strains. Genotype 2 was detected in one patient. Although the sample size was small, the results suggested that genotypes 1 and 3 might be equally distributed. The presence of genotypes 2 and 3 has not previously been appreciated in Brazil, probably due to the lack of an assay able to detect and distinguish all known erythrovirus variants. This work provides the first evidence of the presence of erythrovirus genotypes 2 and 3 in South America, and the results extend our understanding of the distribution of these variants in Brazil.

There is a paucity of information on the clinical courses of patients with genotype 2 and 3 viruses. In this study we showed that parvovirus B19-related symptoms appeared in patients infected with the other genotypes. This was not surprising, since the 12 patients with positive erythrovirus DNA had been selected for the study based on clinical manifestations suggestive of erythrovirus genotype 1 infection. However, detection of genotype 2 and 3 strains in our subjects does not necessarily mean that these viruses are directly linked to the pathogenesis of illness. As no serological results were available for the subjects, the viral infection in our patients may be persistent (12) rather than active. The evidence for persistence of erythrovirus genotype 1 DNA in bone marrow from a previous report (3) may point to the need for further studies to evaluate the prevalence and to assess the clinical significance of other erythrovirus variants in bone marrow samples from asymptomatic individuals.

Nucleotide sequence accession numbers. The sequences of our isolates were reported to GenBank under accession numbers DQ229350 to DQ229361.

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