

# Molecular characterization of human erythrovirus B19 strains obtained from patients with several clinical presentations in the Amazon region of Brazil

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## Abstract

**Background:** Human erythrovirus B19, endemic in the Amazon region since 1990, is associated with a wide spectrum of clinical presentations. **Objectives:** To assess the prevalence of erythrovirus B19 infection and the relative frequency of erythrovirus B19 genotypes in patients in the Amazon region with various clinical presentations.

**Study design:** A total of 487 clinical samples obtained from patients with symptoms suggestive of erythrovirus infection were tested using specific IgM and IgG antibody assays (ELISA) and PCR for viral DNA detection. Partial VP1 and VP2 regions were sequenced and genotyped by phylogenetic reconstruction.

**Results:** B19 DNA was detected in 117 (24%) of 487 samples. Of these, 106 (91%) isolates were genotype 1 and 11 (9%) were genotype 3. No genotype 2 was found. Genotype 1 had three clusters (A1, A2 and B) and all genotype 3 sequences were subtype 3b. All patients with hematological disorders within cluster B of genotype 1 were infected by the same B19 lineage, suggesting that this lineage of B19 may have been transmitted via transfusion of blood products.

**Conclusion:** We reported two genotypes, 1 and 3b, with three genotype 1 clusters co-circulating in the Amazon region during the past 10 years.

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**Keywords:** Erythrovirus B19; Genotypes 1 and 3; Clinical manifestations; Phylogenetic analysis

## 1. Introduction

Human parvovirus B19 has a worldwide distribution and is associated with several clinical illnesses, including erythema infectiosum (EI), transient aplastic crisis (TAC), arthritis, persistent anemia in immune compromised patients and hydrops fetalis (Chorba et al., 1986; Caul et al., 1988; Kurtzman et

al., 1988; Anderson, 1990; Cubel et al., 1992; Foto et al., 1993; Ueno et al., 1993; Cassinotti et al., 1995; Kajigaya and Momoeda, 1997; Koduri, 2000; Chisaka et al., 2006). Human erythroviruses are grouped into three distinct genotypes: genotype 1 (B19 strains), genotype 2 (A6 strains) and genotype 3 (V9/D91.1 strains) (Servant et al., 2002). Parsyan et al. (2007) have proposed two distinct subtypes of genotype 3, 3a and 3b, related to strains V9 and D91.1, respectively. In Brazil, the association of B19 infection with EI was reported 20 years ago (Freitas et al., 1988; Miranda et al., 1989). Early

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studies in Rio de Janeiro detected B19 in the plasma of healthy blood donors (Cruz et al., 1989). Extensive serological surveys among both urban and remote Amazonian communities have revealed a wide range of prevalence rates (Freitas et al., 1990, 1993, 1999, 2002). In this report, we describe the prevalence and relative frequency of erythrovirus genotypes in sera of patients with distinct clinical conditions in the Amazon region.

## 2. Material and methods

### 2.1. Clinical samples

Samples were obtained in the Brazilian Amazon, between January 1995 and December 2005. A total of 487 blood samples were collected from individuals with exanthematous illnesses (EIs) (group I) ( $n=207$ ), hematological disorders (HDs) (group II) ( $n=188$ ), arthropathy (AP) (group III) ( $n=67$ ) and novel B19-associated conditions including encephalitis, hepatitis, myocarditis (MC) and systemic lupus erythematosus (LEs) (group IV) ( $n=25$ ). Of the 487 individuals, 197 were male. Patient age ranged from 1 month to 80 years (median 18 years). Blood samples were collected and stored according to Freitas et al., 2007. The study was approval by the ethical review boards from IEC and the University of São Paulo (CEPSH-ICB-USP).

### 2.2. Serological assay

Detection of IgM- and IgG-specific antibodies to erythrovirus B19 was performed using a commercial enzyme immune-assay manufactured by Biotrin™, Dublin, Ireland, according to the manufacturer's instructions.

### 2.3. PCR detection and sequencing

The partial VP1/VP2 region of the B19 genome was amplified as reported by Durigon et al. (1993). Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit on an ABI 3100 automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA).

### 2.4. Phylogenetic analysis

Sequences were aligned using the BioEdit Editor version 7.0.5.2 (Hall, 2004). Maximum likelihood (ML) phylogenetic tree was generated using PAUP\* v4.0b10 (Swofford, 2003), by the tree bisection-reconnection (TBR), subtree pruning regrafting (SPR) and the nearest-neighbor interchange (NNI) heuristic methods. The HKY+ $\Gamma$  was the substitution model used as the best fitted by Modeltest 3.7 (Posada and Crandall, 1998). The same model was also used to estimate maximum a posteriori (MAP) trees using a Bayesian Markov Chain Monte Carlo (MCMC) method

available in the BEAST v1.6 package, with chain length of 40 million (Drummond and Rambaut, 2007). The MCMC method was also used to estimate the time of introduction of genotype 3 into the Amazon region, under the assumption of a relaxed molecular clock and several different models of demographic history: constant population size, exponential population growth, logistic growth, expansion growth, and a Bayesian skyline plot. Sampling dates for the reference sequences were obtained from Parsyan et al. (2007).

We used 117 sequences generated here (GenBank under accession numbers EF089178–EF089196, EF089198–EF089232 and EU142742–EU142804), including a previously published dataset with 46 sequences from genotype 1 from Belém (Freitas et al., 2007). The reference sequences had the following accession numbers: genotype 1 (U31358, U38507, U38509, U38510, U38515, U38546, U53600, AB030673, AB126271, AF161224, AF162273, AY386330, AY028237, M13178 and Z70528); genotype 2 (AY044266, AY044268, AY064476 and AY064475); genotype 3a (DQ234769, DQ234771, AY582125 and NC\_004295); genotype 3b (AY083234, AY582124, AY647977, DQ234779 and DQ234778). Therefore, a total of 145 sequences were compiled for analysis.

## 3. Results

Of the 487 samples examined serologically, 363 (74.5%) were B19 antibody-positive and 124 (25.5%) were antibody-negative (Fig. 1). Erythrovirus DNA was amplified and sequenced from 117 of the 487 samples (24%) (Fig. 1 and Table 1). Of the 117 isolates amplified, 107 (91%) had IgM antibodies only, 3 (2.6%) had IgG antibodies only, and 7 (6%) had neither IgM nor IgG antibodies (Table 1). It was presumed that samples positive for IgM and/or IgG antibodies, but DNA-negative were derived from patients in whom viraemia was no longer present or was at undetectable levels. The seven samples negative for IgM and/or IgG antibodies but positive by DNA testing were obtained from patients with erythema infectiosum ( $n=5$ ) and haematological disorders ( $n=2$ ) that had been collected 3–5 days after the onset of

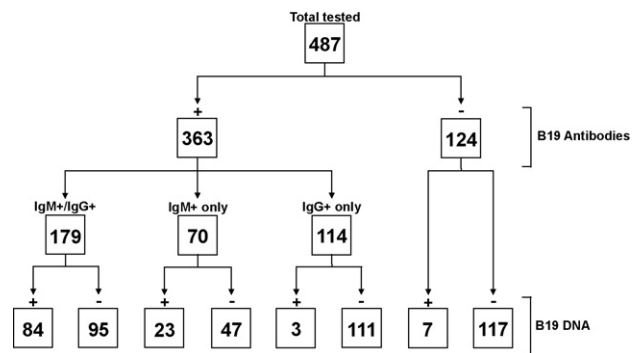


Fig. 1. Serological and virological laboratory investigations in samples from 487 patients in the Amazon region of Brazil.

Table 1  
Clinical features and laboratory results from patients infected with erythrovirus in the Amazon region, 1995–2005

Clinical group	Age group	Serological status/DNA detection/genotype												Total (N = 117)		
		IgM <sup>+</sup> <sup>a</sup>						IgM <sup>-</sup> /IgG <sup>-</sup>						DNA <sup>+</sup>	G1	G3
		DNA <sup>+</sup>	G1	G3	DNA <sup>+</sup> only	DNA <sup>+</sup>	G1	G3	DNA <sup>+</sup>	G1	G3					
I	≤15	54	48	6	1	1	0	4	4	4	0	0	59	53 (45.3%) <sup>a</sup>	6 (5%)	
	>15	8	8	0	0	0	0	1	1	1	0	0	9	9 (7.7%) <sup>b</sup>	0 (0%)	
II	≤15	13	13	0	2	2	0	2	1	1	1	0	17	16 (13.7%)	1 (0.8%)	
	>15	16	15	1	0	0	0	0	0	0	0	0	16	15 (12.8%)	1 (0.8%)	
III	≤15	4	2	2	0	0	0	0	0	0	0	0	4	2 (1.7%) <sup>c</sup>	2 (1.7%)	
	>15	10	10	0	0	0	0	0	0	0	0	0	10	10 (8.5%) <sup>d</sup>	0 (0%)	
IV	≤15	2	1	1	0	0	0	0	0	0	0	0	2	1 (0.8%)	1 (0.8%)	
	>15	0	0	0	0	0	0	0	0	0	0	0	0	0 (0%)	0 (0%)	
Total		107	97	10	3	3	0	7	6	6	1	1	117 (24%)	106 (91%)	11 (9%)	

I = exanthematous illness; II = hematological disorders; III = arthropathy; IV = other clinical conditions; G1 = genotype 1; G3 = genotype 3. a vs. b =  $p < 0.0001$ ; c vs. d =  $p < 0.001$ .

<sup>a</sup> This subgroup includes all samples which reacted with either IgM<sup>+</sup> only or IgM<sup>+</sup>/IgG<sup>+</sup>.

symptoms (Table 1). In group I, 53 (45.3%) genotype 1 strains were found in individuals ≤15 years and 9 (7.7%) in individuals >15 years ( $p < 0.0001$ ). Six (5%) genotype 3 strains were found in individuals ≤15 years. In group III, 2 (1.7%) genotype 1 samples were found in individuals aged ≤15 years; 10 (8.5%) were from those aged >15 years ( $p < 0.001$ ). Two (1.7%) genotype 3 strains were obtained, both from individuals ≤15 years.

The maximum likelihood tree agreed with the MAP tree estimated with BEAST (Fig. 2), where 106 (91%) of 117 isolates clustered within genotype 1 and 11 (9%) clustered into genotype 3. Genotype 1 sequences included three distinct clusters (A1, A2 and B). Cluster A1 included 37 cases of EIs, 13 cases of HDs and 7 cases of AP. In cluster A2 there were 19 with EI, 6 with HD and 3 with AP. Cluster B included 7 with EI, 11 with HD, 2 with AP and 1 with MC. Among patients with genotype 3 there was one case of systemic LE, 6 with EI, 2 with HD and 2 with AP. Importantly, the frequency of HD in cluster B (52%) was significantly higher ( $\chi^2 = 7.48$ ,  $p < 0.006$ ) than the frequency of HD in clusters A1 and A2 (22% and 21%, respectively).

Both the evolutionary rate and the dates of divergence for genotype 3 are shown in Table 2. The best-fit model indicated a logistic population growth with a mean nucleotide substitution rate of  $1.2 \times 10^{-3}$  (95% HPD of  $3.10 \times 10^{-4}$  to  $2.32 \times 10^{-3}$ ) (HPD, high probability density). Given this rate, the most recent common ancestor (MRCA) (*i.e.*, the time of the oldest node in the tree, indicating the age of the descendent lineage) for all sequences from genotype 3 was 36 years (95% HPD 14–52 years). The mean time of origin of subtypes 3a and 3b was 21 (95% HPD 10–41 years) and 28 (95% HPD 14–49 years), respectively. The MRCA for genotype 3b in Belém was 19 years (95% HPD 9–38 years).

#### 4. Discussion

Until now, there has been only a few studies reporting the prevalence of erythrovirus genotype 1 (B19-type virus) and 3 (V9 and D91.1-related strains) in temperate and tropical countries (Heegaard et al., 2001; Servant et al., 2002; Sanabani et al., 2006; Toan et al., 2006). Ours is the first report on these distinct genotypes in the Amazon region. The predominance of genotype 1 (B19) (91%) and the relatively low frequency of genotype 3 (9%) in our study are similar to those previously found in Vietnam (Toan et al., 2006) and France (Servant et al., 2002). The high frequency of genotype 1 may be explained by the earlier introduction of B19-type virus lineages in the Amazon region about 45 years ago (Freitas et al., 2007). Interestingly our data suggest a recent introduction of the D91.1 variant of genotype 3 in the Amazon (19 years ago) compared to estimates elsewhere with an MRCA of 36 years in the past (Table 2).

Sequences from genotypes 1 and 3b had average genetic diversities of 1.1% and 0.8%, respectively, and diverged 12.8% between genotypes. These values are consistent with

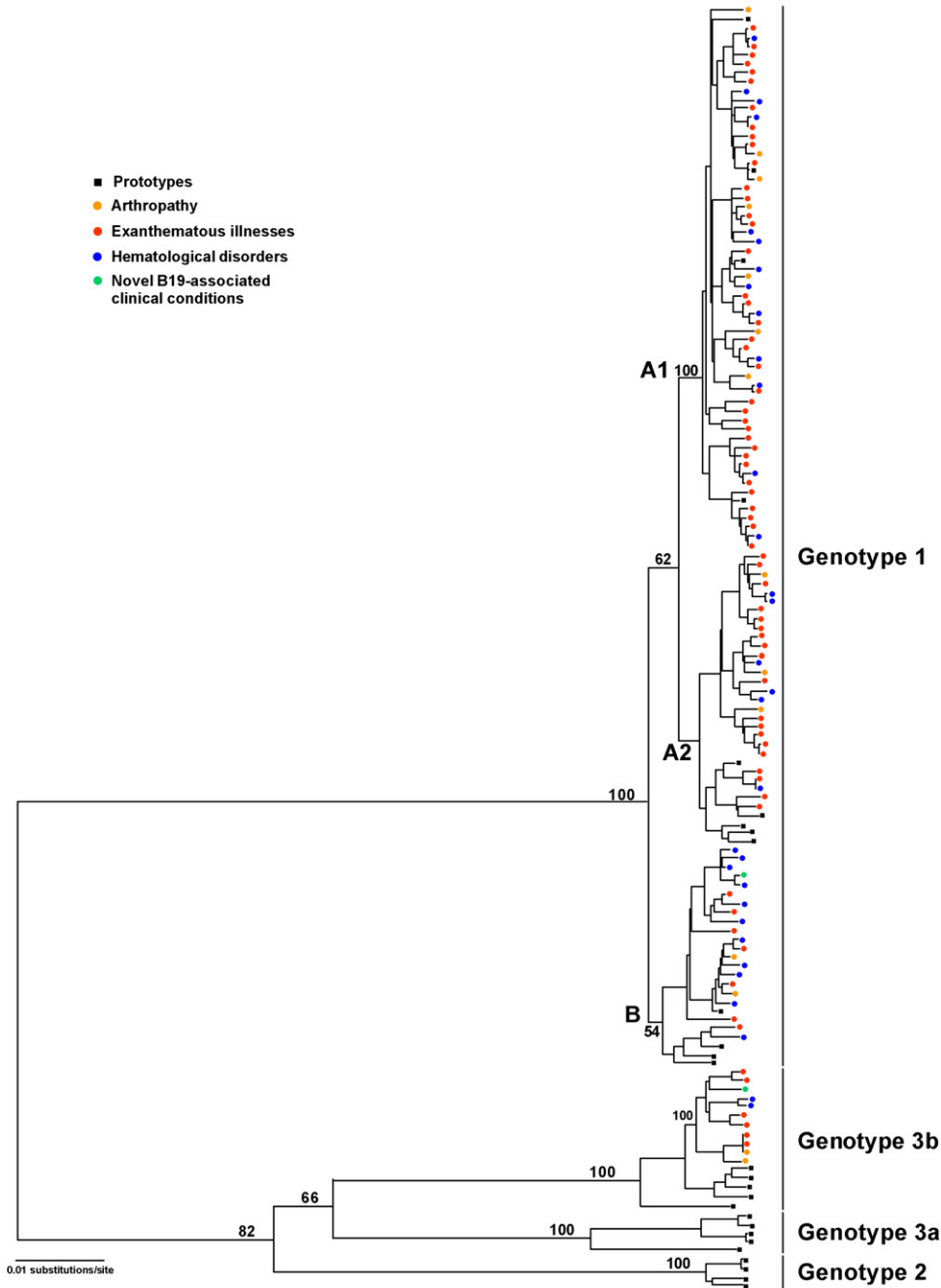


Fig. 2. Maximum a posteriori (MAP) tree of partial region (476 bp) of B19 virus VP1/VP2 region, obtained in 117 patients from North region of Brazil, from January 1995 to October 2005. Prototypes of the three B19 genotypes were presented in the tree, together with strains from other places. Posterior values are specified on the nodes. Scale bar is proportional to the genetic distance.

previous studies in which the same region of the VP1/VP2 gene was analyzed (Servant et al., 2002; Toan et al., 2006; Parsyan, et al., 2007). The mean substitution rate of  $1.2 \times 10^{-3}$  nucleotide substitution/(site year) we found in genotype 3 strains was higher than rates previously reported for genotypes 1 (Shackleton and Holmes, 2006; Freitas et al., 2007) and 3 (Parsyan et al., 2007). Using this rate, the MRCA for genotype 3 was dated 36 years ago (95%

HPD 14–52 years). Our results differ considerably from the MRCA estimate of 525 years ago found by Parsyan et al. (2007). However, the 95% HPD values of this estimate were extremely large (95% HPD 43.5–1992 years) and overlapped with our estimate (95% HPD 14–52 years). It appears that the introduction of genotype 3b into the Amazon region occurred recently (19 years ago, 95% HPD of 9–38 years). The mean genetic divergence of 0.8% within the Amazon 3b

Table 2

Bayesian estimates of population dynamics and evolutionary parameters for the VP1/VP2 region of human erythrovirus D91.1 (genotype 3)

	Genotype			
	All 3	3a	3b all	3b Belém
Number of samples	21	5	16	11
Range of sample collection	1991–2005	1995–2005	1991–2004	1997–2003
Molecular clock type	Relaxed	NA	NA	NA
Best-fit demographic model	Logistic growth	NA	NA	NA
Substitution rate <sup>a</sup> (95% HPD <sup>b</sup> )	$1.2 \times 10^{-3}$ ( $3.1 \times 10^{-4}$ to $2.32 \times 10^{-3}$ )	NA	NA	NA
Mean age (years) of MRCA <sup>c</sup> (95% HPD)	36 (14–52)	21 (10–41)	28 (14–49)	19 (9–38)
Mean rate of population growth <sup>d</sup> (95% HPD)	3.89 ( $1.034 \times 10^{-4}$ to 9.228)	NA	NA	NA
Mean epidemic doubling time, $\lambda^e$ (years)	5.44	NA	NA	NA

NA, not applicable.

<sup>a</sup> Mean number of nucleotide substitutions per site, per year.<sup>b</sup> High probability density.<sup>c</sup> Most recent common ancestor.<sup>d</sup> Mean number of new infections per host, per year.<sup>e</sup> Time required for the effective number of infections to double in size ( $\lambda = \ln(2)/r$ , where  $r$  = growth rate).

sequences was consistent with a single recent introduction and is almost three times lower than that of subtype B19/3a in Ghana (2.72%) (Parsyan et al., 2007).

As in studies from Vietnam, we observed a higher frequency of genotype 1 associated with EI in children (45.3%) than adults (7.6%) (Toan et al., 2006). However, in AP patients, genotype 1 was more frequent in adults (8.5%) than children (1.7%). Nearly identical age distribution was observed in HD patients (children, 13.7% and adults, 12.8%).

Similar to the findings of Servant et al. (2002) we found that genotype 3 was associated with both a wider range of clinical manifestations and an uneven distribution within clinical groups, with a higher frequency among children with EI. Only one adult infected with genotype 3 in our study had HD, an apparent difference between our results and previous studies reporting a higher frequency of genotype 3 in adult patients with hematological symptoms (Nguyen et al., 1999; Servant et al., 2002; Sanabani et al., 2006).

In genotype 1 we found three different clusters (A1, A2 and B) with elevated rates of non-synonymous substitutions after being independently introduced in the Amazon region (Freitas et al., 2007). Interestingly, genotype 1 was more frequently found in women in three of the four clinical groups (data not shown), corroborating previous findings from patients with AP in the Amazon (Freitas et al., 2002). Moreover, we observed a statistically higher proportion of patients within cluster B with HD than in clusters A1 or A2.

All samples from cluster B associated with HD patients came from the local blood bank, the majority having been collected between 2004 and 2005. Moreover, the basal lineage of cluster B was isolated from a patient receiving blood products in Rio de Janeiro in 1988, possibly indicating that cluster B viruses have been associated with blood product therapy for a significant time. These worrisome findings warrant further investigation as to whether HD patients might have acquired infection from the same B19 lineage (cluster B). It should be pointed out that the Brazilian health system has no routine

screening for B19 in blood banks and B19 can be transmitted by blood products (Beersma et al., 2005).

In summary, we found two genotypes, 1 and 3b, circulating in the Amazon region of Brazil and three genotype 1 clusters co-circulating during the past 10 years. Prospective studies are needed to monitor the frequency of erythrovirus genotypes in the Amazon region and the possible appearance of genotype 2. We believe that our data should be taken as a warning by the Brazilian Health System to consider including B19 screening in blood banks. Besides the primary respiratory route of transmission, our findings lead to the hypothesis that B19 may also have been transmitted for some years through the administration of blood products to susceptible patients.

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