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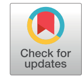
Introduction of human gammaherpesvirus 8 genotypes A, B, and C into Brazil from multiple geographic regions



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ABSTRACT

Variations in the open reading frame (ORF) K1 gene sequence of human gammaherpesvirus 8 (HHV-8) has led to the identification of 6 major genotypic clades (A, B, C, D, E, and F) in specimens isolated from around the world. These clades exhibit clear clustering among individuals in different ethnic groups and from different geographic regions. The human population of Brazil varies greatly in ethnicity because of multiple immigration events from Africa, Europe, Asia, and indigenous communities. However, there is scant information about the HHV-8 genotypes currently circulating in Brazil. Here, we describe HHV-8 genotypic diversity in isolates from Brazilian HIV-infected patients living with Kaposi's sarcoma (KS) by analysis of the complete ORF-K1 region. We also identified the most likely geographic origins of these different Brazilian genotypes. We extracted HHV-8 DNA (24 positive samples) from individuals with HIV/KS from the states of São Paulo and Rio de Janeiro, amplified the ORF-K1 gene using nested PCR (about 870 base pairs), performed sequencing and phylogenetic analysis, and then calculated the mean genetic distances of Brazilian sequences from sequences in other regions of the world (523 sequences analyzed). Phylogenetic analysis showed that genotypes C, A, and B were present in 45.8 %, 29.2 % and 25 % of the isolates from Brazil, respectively. These isolates grouped into separate clades, rather than a single monophyletic cluster. Mean genetic distance analyses suggested that these genotypes were introduced into the Brazil multiple times from different geographical regions. HHV-8/A isolates appear to be from Ukraine, Russia, and the Tartar ethnic group; HHV-8/B isolates appear to be from Congo and Democratic Republic of the Congo; and HHV-8/C isolates appear to be from Australia, Algeria, England, and French Guiana. These results contribute to a better understanding of the genetic diversity and origins of HHV-8 strains circulating in Brazil, and will provide a foundation for further epidemiological and evolutionary studies of HHV-8.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also called human gammaherpesvirus 8 (HHV-8), is in the Herpesviridae family, the Gammaherpesvirinae subfamily, and the Rhadinovirus genus. This virus is the etiologic agent for all forms of the vascular neoplasm known as Kaposi's sarcoma (KS), a cancer that is common in patients with HIV/AIDS (Pérez and Tous, 2017).

HHV-8 has a highly conserved double-stranded DNA genome of approximately 140 kilobases. However, both ends of the genome show significant variability, allowing categorization of HHV-8 into different genotypes (Isaacs et al., 2016; Olp et al., 2015). The 5′end has a highly variable open reading frame (ORF) of about 870 base pairs (bp) (called ORF-K1) that codes for a transmembrane protein composed of 289 amino acid (aa) residues. ORF-K1 has multiple roles in cellular signal transduction, viral reactivation, endothelial cell immortalization, and

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Table 1
Primers, probe, and synthetic standard used for detection and quantification of HHV-8.

HHV-8	Sequence (5´→3´)
Primers	Forward – GGGCCCCGGATGATGTA
	Reverse – GCCCCATAAATGACACATTGG
Probe	FAM-AGATCAAGTTCCGCCATAT-MGB
Synthetic standard	TTCGTGCCCCATAAATGACACATTGGCGTATATATGGCGGAACTTGATCTATGCGTTACATCATCCGGGGCCCCTGATA

HHV-8: Human gammaherpesvirus 8, A: adenine, T: thymine, C: cytosine, G: guanine, FAM: 5(6)-carboxyfluorescein, MGB: minor groove binder.

host immune recognition (Lee et al., 2003).

Phylogenetic studies based on the highly variable HHV-8 ORF-K1 identified six major genotypes (A, B, C, D, E, and F) whose sequences differ by up to 30 % at the aa level (Isaacs et al., 2016; Olp et al., 2015). These different genotypes have variable penetrance in different human populations, and all exhibit clear clustering with different ethnic and geographic groups, possibly due to ancient human migrations (Isaacs et al., 2016). Thus, genotypes A and C occur predominantly in Europe, the United States, most parts of Asia, and the Middle East; genotype B is predominant in Africa; the rare genotype D was only reported in the Pacific islands and Taiwan; genotype E was reported in Amerindian populations of Brazil, French Guiana, and Ecuador; and genotype F, the most recently identified form, was reported in Uganda and Brazil (Tozetto-Mendoza et al., 2016).

Brazil has significant ethnic diversity because of multiple migratory flows from Africa, Europe, Asia, and indigenous communities, but there is scant information about HHV-8 genetic diversity in Brazil. The aim of this study was to characterize HHV-8 isolates from HIV/KS patients living in Brazil based on phylogenetic analysis of the complete ORF-K1 gene.

2. Materials and methods

2.1. Ethics statement

The present study protocol was approved by the Ethics and Research Committee of the School of Medicine from São Paulo University and Emílio Ribas Infectology Institute (number 1.560.798), and the Oswaldo Cruz Foundation Ethics Committee (number 0032.0.009.000-1). This study also complied with the ethical standards of the Declaration of Helsinki. All patients signed written informed consent agreements before participation. Clinical and epidemiological informations were collected from medical records.

2.2. Samples

This study was retrospective, and fourteen saliva samples were collected between August 2016 and February 2018 from HIV/KS patients who were enrolled at the outpatient clinic of the HIV/AIDS Patient Care Extension Service (SEAP HIV/AIDS) of the Clinical Division of Infectious and Parasitic Diseases, University of São Paulo-School of Medicine Clinical Hospital (ICHCFMUSP), and at Dia Hospital, Emílio Ribas Infectology Institute, São Paulo City. Ten serum samples were collected between January 2017 and August 2018 from HIV/KS patients who were enrolled at the National Institute of Infectology Evandro Chagas (INI), Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro City. Both Institutes are referral centers for the care and treatment of individuals with HIV/KS in these states.

As saliva collection from HIV/KS patients is routine in these hospitals from São Paulo, we chose these samples for HHV-8 detection. However, this collection is not routine at the Evandro Chagas National Institute of Infectious Diseases from Rio de Janeiro. For this reason, we obtained serum samples from patients with HIV/KS for this detection. All HIV-infected patients received combined antiretroviral therapy.

Inclusion criteria were as follows: HIV infection confirmed using enzyme-linked immunosorbent assay, KS confirmed by biopsy, any gender, ethnicity/color, age, CD4 + T lymphocyte count (CD4+ Count), and HIV load. The variables used were: gender (female, male), ethnicity/color (white, black/ brown/mulatto), age (20–29 years,30–39 years, 40–49 years, and ≥ 50 years), CD4+ Count (< 200 cells/mm^3 of blood, 200 e 350 cells/mm^3 of blood, 351 e 500 cells/mm^3 of blood), and HIV load (Mean ± SD in copies/mL).

Saliva samples were collected as described by Beyari et al. (Beyari et al., 2003) and Tozetto-Mendoza (Tozetto-Mendoza et al., 2018), and stored at −70 °C until analyses. Serum samples were collected and stored at −20 °C until analyses.

Viral DNA was extracted from homogenized samples using the High Pure Viral Nucleic Acid Kit (Roche, New Jersey, EUA) according to the manufacturer's instructions. DNA was stored at −70 °C until analyses. The quantity and quality of DNA samples were assessed as previously described (Saiki et al., 1985).

All samples were suitable for viral DNA amplification. A quantitative (real-time) polymerase chain reaction (qPCR) with HHV-8 ORF26-specific primers and a probe was performed to confirm the presence of HHV-8 in all samples, as reported previously (Levi et al., 2011). In addition, a synthetic standard curve (Lopes et al., 2019), with initial titer of 10^7 copies/mL and a dilution range of 10 to 10^7 was used for this qPCR assay (Table 1). All samples were tested in triplicate, and all samples were successfully amplified. Results are expressed as copies per mL.

2.3. ORF-K1 amplification

The ORF-K1 sequence (about 870 bp) was amplified to identify HHV-8 genotypes. PCR reactions and cycling parameters were adapted from Lacoste et al. (Lacoste et al., 2000a) and Poole et al. (Poole et al., 1999) and were according to the protocol of Mendoza et al. (Tozetto-Mendoza et al., 2016).

Briefly, semi-nested PCR reactions were performed in a 25 μL reaction mixture containing 100–200 ng of DNA template, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer (Table 2), 1X of 10XPCR buffer minus Mg, and 1 U/μL of Platinum Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA).

The PCR thermal conditions for the first and second rounds were as follows: initial 3 min denaturation at 95 °C; 45 cycles of 95 °C for 30 s, 61.5 °C for 50 s, and 72 °C for 1 min; and a final extension at 72 °C for 3 min. PCR products were visualized on 1.5 % agarose gels that were stained with ethidium bromide. A UV transilluminator and the Low DNA Mass Ladder (Gibco) were used to identify PCR products.

All laboratory procedures were performed under stringent conditions to avoid contamination. Four separate rooms were used, one for extraction, another to prepare amplification mixes, another to apply DNA to the mix tubes, and a fourth room where amplicons were handled. DNAs isolated from saliva and serum samples that were HHV-8 positive and negative were used as controls.

2.4. ORF-K1 purification and sequencing

ORF-K1 PCR products were purified using the High Pure PCR Product Purification kit (Roche, New Jersey, EUA) according to the

Table 2
Primers used in amplification and sequencing of HHV-8 ORF-K1.

Round PCR	Primers	Sequences (5′-3′)	Nucleotide positions[a]	References
First	Forward LGH2089	GTTCTGCCAGGCATAGTC	21-38	Poole et al., 1999
First	Reverse K1AG1200AS	AGGCCATGCTGTAAGTAGCACGGTT	1094-1118	Lacoste et al., 2000a,b
Second	Forward LGH2089	GTTCTGCCAGGCATAGTC	21-38	Poole et al., 1999
Second	Reverse LGH2088	AATAAGTATCCGACCTCAT	1037-1055	Poole et al., 1999

[a] NCBI Reference Sequence: NC_009333.1.

Table 3
Clinical and epidemiological characteristics of HIV/KS patients living in São Paulo and Rio de Janeiro, Brazil.

Variables	Total (N = 24)		São Paulo (N = 14)		Rio de Janeiro (N = 10)		*P-value*[#]
	n	%	n	%	n	%	
Gender							
Female	01	4.2	00	00	01	10.0	–
Male	23	95.8	14	100	09	90.0	
Ethnicity/color							
White	07	29.2	04	28.6	03	30.0	0.939
Black/Brown/mulatto	17	70.8	10	71.4	07	70.0	
Age							
20-29 years	06	25.0	03	21.4	03	30.0	0.297
30-39 years	05	20.8	04	28.6	01	10.0	
40–49 years	08	33.3	03	21.4	05	50.0	
≥ 50 years	05	20.8	04	28.6	01	10.0	
*CD4+ Count (cells/mm^3)***							
< 200	14	63.6	08	57.1	06	75.0	–
200 e 350	06	27.3	04	28.6	02	25.0	
351 e 500	02	9.1	02	14.3	00	0.0	
	Mean ± SD		**Mean ± SD**		**Mean ± SD**		***P-value***[§]
HIV load (copies/mL)	$1.65*10^5 \pm 1.96*10^5$		$1.64*10^5 \pm 1.74*10^5$		$1.69*10^5 \pm 2.43*10^5$		0.962
HHV-8 load (copies/mL)	$5.29*10^8 \pm 1.59*10^9$		$1.59*10^7 \pm 3.87*10^7$		$1.25*10^9 \pm 2.34*10^9$		0.131

Note: N, number of participants; **Missing variables; [#] Chi-square test, Fisher's exact test; [§]Student's *t*-test; SD, standard deviation.

manufacturer's instructions. Purification products were visualized on 1.5 % agarose gels that were stained with ethidium bromide. A UV transilluminator and the Low DNA Mass Ladder (Gibco) were used to identify purification products.

The DNA sequencing of purified PCR products (~20 ng) was performed at the Platform of Technological Development Program in Materials for Health (PDTIS) of Fiocruz (Oswaldo Cruz Foundation, Rio de Janeiro) using the ABI Kit BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions, and ABI 3730xl Automated Sequencers (Applied Biosystems) for both complementary strands. Second-round semi-nested PCR primers were used during this stage (Table 1).

2.5. Phylogenetic analysis

Fifty-nine complete HHV-8 ORF-K1 sequences from GenBank (http://www.ncbi.nlm.nih.gov/) were used as references for analysis. Reference sequences and Brazilian sequences from this study were aligned using MUSCLE software (Edgar, 2004), and manually edited with the MEGA program, version 7 (Kumar et al., 2016). A phylogenetic tree was constructed in MEGA using the maximum likelihood method (ML), with the GTR + G+I model of rate heterogeneity. Bootstrapping was performed with 500 replicates, and a bootstrap value above 70 % was used to confirm the robustness of each major branch.

2.6. Mean genetic distance

The mean genetic distances between complete ORF-K1 sequences from Brazil and other regions of the world were examined to identify the possible geographic origins of the genotypes identified in this study. A total of 523 complete ORF-K1 sequences that corresponded to genotypes A, B, and C and were deposited in GenBank were collected for these analyzes. The mean genetic distances between groups were estimated using MEGA, version 7 (Kumar et al., 2016), with the Kimura two-parameter model (Kimura, 1980).

2.7. Statistical analysis

Descriptive statistics of the qualitative variables was determined by frequency distribution. Afterwards, the Chi-square test was used for categorical variables at 95 % confidence intervals (CIs) and p value ≤ 0.05 to compare proportions between Sao Paulo and Rio de Janeiro groups. The Student's *t*-test was used to compare the mean (SD) between Sao Paulo and Rio de Janeiro groups also using 95 % CI and p-value ≤0.05.One-Way ANOVA was used to compare the viral load of HIV and HHV8 between the three genotypes (A, B and C), followed by Tukey's test for multiple comparisons between means (SD), 95 % CI and p-value ≤0.05.

3. Results

The clinical and epidemiological characteristics of 24 HIV/KS patients living in São Paulo and Rio de Janeiro are presented in Table 3. We did not find any statistical difference between these groups, probably due to the small sample size.

We successfully amplified all samples for sequencing of the

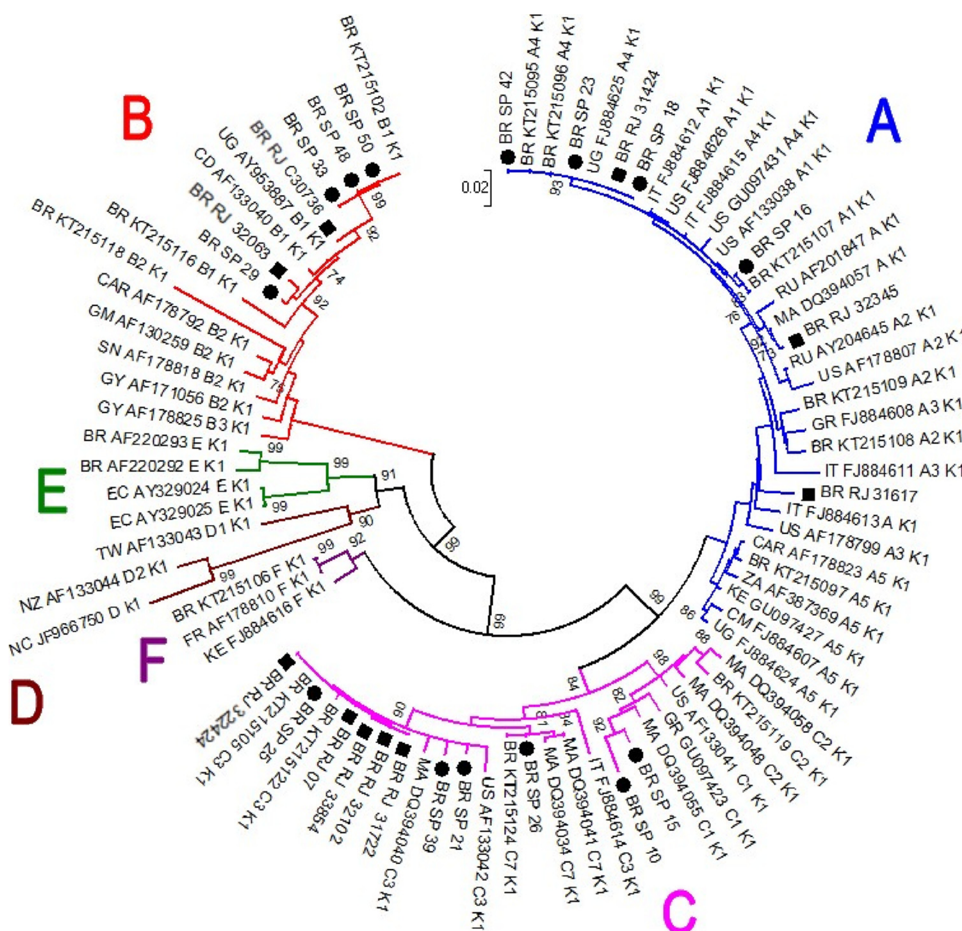

Fig. 1. Phylogenetic analysis of HHV-8 ORF-K1 sequences using the maximum-likelihood method. GenBank accession numbers for the reference sequences are: genotype A, KT215095, KT215096, FJ884625, FJ884612, FJ884626, FJ884615, GU097431, AF133038, KT215107, AF201847, DQ394057, AY204645, AF178807, KT215109, FJ884608, KT215108, FJ884611, FJ884613, AF178799, AF178823, KT215097, AF387369, GU097427, FJ884607, FJ884624; genotype B, AF178825, AF171056, AF178818, AF130259, AF178792, KT215118, KT215116, AF133040, AY953887, KT215102; genotype C, DQ394058, KT215119, DQ394048, AF133041, GU097423, DQ394055, FJ884614, DQ394041, DQ394034, KT215124, AF133042, DQ394040, KT215122, KT215105; genotype D, JF966750, AF133043, AF133044; genotype E, AY329025, AY329024, AF220293, AF220292; and genotype F, FJ884616, AF178810, KT215106. The HHV-8 genotypes are indicated in the figure. Reference sequences are indicated by the country or origin (BR, Brazil; CAR, Central African Republic; CD, Democratic Republic of the Congo; CM, Cameroon; EC, Ecuador; FR, France; GM, Gambia; GY, Guiana; GR, Greece; IT, Italy; KE, Kenya; MA, Morocco; NC, New Caledonia; NZ, New Zealand; RU, Russia; SN, Senegal; TW, Taiwan; UG, Uganda; US, United States of America; ZA, South Africa) followed by accession number, genotype, and gene sequenced (ORF-K1). New sequences from this study are denoted "BR", followed by the location and code, and a symbol indicating location within Brazil (filled circle: São Paulo, SP; filled square: Rio de Janeiro, RJ). Values at internal nodes indicate percentages of 500 bootstrap replicates that support the branch.

complete HHV-8 ORF-K1 sequence. Fig. 1 shows the phylogenetic reconstruction of new sequences from the 24 HIV/KS individuals from Brazil (including 14 from the state of São Paulo and 10 from the state of Rio de Janeiro), and 59 reference sequences from GenBank.

This phylogenetic analysis (Fig. 1) clearly distinguishes the six known genotypic clades (A, B, C, D, E, F), and all these branches have high bootstrap values. Among all of our 24 samples from Brazil, genotype C was predominant (11, 45.8 %), followed by genotype A (7, 29.2 %) and genotype B (6, 25 %). The proportion of genotypes A, B, and C were 28.6 %, 28.6 % and 42.8 % in the SP group, and 30 %, 20 % and 50 % in the RJ group. By comparing the genotypic distribution of HHV-8 between the states of São Paulo and Rio de Janeiro, no statistical difference was observed (Chi-square test, Fisher's exact test, $P = 0.885$).

There was no statistical difference in the genotypic distribution of HHV-8 between clinical and epidemiological characteristics (Chi-square test, p-value > 0.05). No statistical difference was observed in the genotypic distribution of HHV-8 between viral load of HHV-8 and HIV (One-Way ANOVA, Tukey's test, $P = 0.852$ and $P = 0.531$, respectively).

Table 4 summarizes the HHV-8 data of each of the 24 samples collected from Brazil, including clinical samples, year of collection, viral load, genotype, and GenBank accession number.

We also calculated mean genetic distances between ORF-K1 sequences from different geographical regions (Supplementary Table) and Brazil using Kimura's two parameter model (Table 5). This distance ranged from 1.9 to 4.9 % for clade A, 1.9–5.3% for clade B, and 2.0–5.0% for clade C.

The lowest genetic distances for Brazilian HHV-8/A isolates were in

Table 4
Characteristics of HHV-8 samples collected from HIV/KS individuals in Brazil based on phylogenetic analysis of the HHV-8 K1 gene.

Sample code	Clinical samples	Year of collection	Viral load (copies/mL)	Genotype	GenBank accession number
BR SP 10	saliva	2016	2.51×10^6	C	MN318222
BR SP 15	saliva	2016	1.13×10^5	C	MN318223
BR SP 16	saliva	2016	3.52×10^6	A	MN318207
BR SP 18	saliva	2016	6.59×10^5	A	MN318208
BR SP 21	saliva	2016	2.72×10^5	C	MN318224
BR SP 23	saliva	2016	5.56×10^6	A	MN318209
BR SP 25	saliva	2016	9.29×10^5	C	MN318225
BR SP 26	saliva	2016	9.71×10^3	C	MN318226
BR SP 29	saliva	2016	5.71×10^2	B	MN318213
BR RJ 322424	serum	2017	2.9×10^7	C	MN318220
BR RJ 30736	serum	2017	4.4×10^2	B	MN318212
BR RJ 32063	serum	2017	3.6×10^3	B	MN318211
BR RJ 31722	serum	2017	2.26×10^8	C	MN318219
BR RJ 32345	serum	2017	3.6×10^5	A	MN318206
BR RJ 07	serum	2017	3.8×10^7	C	MN318221
BR RJ 32103	serum	2017	3.55×10^7	C	MN318218
BR SP 33	saliva	2017	3.52×10^6	B	MN318214
BR SP 39	saliva	2017	8.27×10^6	C	MN318227
BR SP 42	saliva	2017	1.22×10^5	A	MN318210
BR SP 48	saliva	2017	5.81×10^7	B	MN318215
BR RJ 31424	serum	2018	7.09×10^9	A	MN318205
BR RJ 31617	serum	2018	9.92×10^7	A	MN318204
BR RJ 33854	serum	2018	1.46×10^9	C	MN318217
BR SP 50	saliva	2018	1.40×10^8	B	MN318216

Table 5
Mean genetic distances between HHV-8 (ORF-K1) isolates from Brazil and other geographic regions.

Country	sequences n	Genotypes					
		A		B		C	
		n	Mean genetic distance	n	Mean genetic distance	n	Mean genetic distance
Algeria	1	0	–	0	–	1	2.1
Argentina	22	3	2.8	4	2.2	15	3.4
Australia	12	9	2.3	0	–	3	2
Botswana	1	1	3.9	0	–	0	–
Cameroon	6	6	3.4	0	–	0	–
Central African Republic	12	5	3.6	5	2.8	2	3.3
Congo	1	0	–	1	1.7	0	–
Cuba	20	14	4.9	5	3.9	1	3.9
Democratic Republic of the Congo	1	0	–	1	1.9	0	–
England	10	5	2.4	1	2	4	2.1
France	14	1	2.8	0	–	13	2.2
French Guiana	11	4	3.2	6	3.4	1	2.1
Gambia	9	0	–	9	4.1	0	–
Greece	8	4	2.9	0	–	4	4.4
Iceland	2	1	2.7	0	–	1	5
Iran	4	0	–	0	–	4	3.4
Italy	30	18	3.1	0	–	12	3.7
Japan	12	5	2.3	0	–	7	2.9
Kenya	13	12	2.9	0	–	1	4.4
Mauritania	1	1	2.7	0	–	0	–
Morocco	35	5	4.4	0	–	30	2.3
Russia	32	26	2.2	0	–	6	3.2
Saudi Arabia	13	10	4.7	0	–	3	3.5
Senegal	2	0	–	1	3.7	1	2.2
South Africa	102	58	3.1	44	3.9	0	–
Spain	2	2	3.1	0	–	0	–
Tartar (Russia)	2	2	1.9	0	–	0	–
Togo	1	0	–	1	5.3	0	–
Tunisia	2	0	–	0	–	2	2.3
Turkey	1	1	2.6	0	–	0	–
Uganda	67	36	3.3	27	3.5	4	4.5
Ukraine	3	2	2.2	0	–	1	3.8
United States	18	12	2.5	0	–	6	3.5
Brazil	53 (24[a])	14 (7[a])	–	20 (6[a])	–	19 (11[a])	–
Total	**523**	**257**		**125**		**141**	

[a] Brazilian HHV-8 sequences generated in this study.

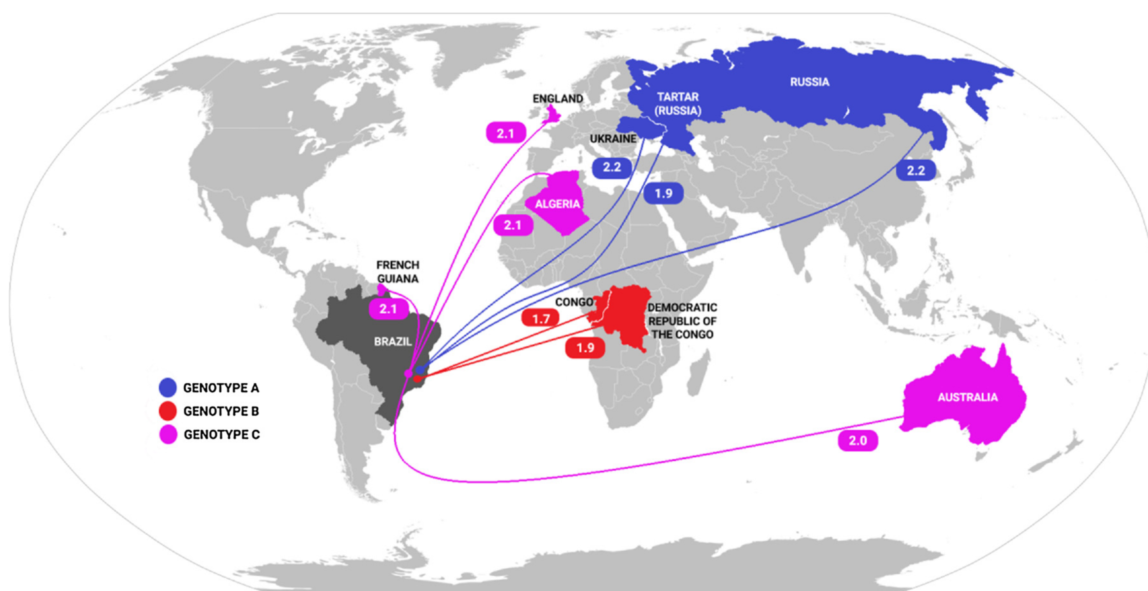

Fig. 2. Possible geographic origins of HHV-8 genotypic clades A, B, and C in Brazil based on calculation of genetic distances from previously described HHV-8 isolates. The lowest mean genetic distance between ORF-K1 sequences from Brazil and other regions are indicated in rectangles. Genotype A: blue; genotype B: red; genotype C: pink.

Ukraine, Russia, and the Tartar ethnic group. The lowest distances for Brazilian HHV-8/B isolates were in Congo and Democratic Republic of the Congo. The lowest distances for Brazilian HHV-8/C isolates were in Australia, Algeria, England, and French Guiana (Table 5). These genetic distance data led to our hypotheses about the possible geographic origins of these different genotypic clades in Brazil (Fig. 2).

4. Discussion

In this study, we characterized isolates of HHV-8 from HIV/KS patients living in Brazil in an effort to better understand the molecular epidemiological characteristics of HHV-8 in this country. The initial molecular classification of HHV-8 strains was based on ORF26 and ORF75 genes. However, analyses using these sequences were limited because of the very low variation of these genes (Fouchard et al., 2000; Pérez and Tous, 2017), which are highly conserved structural genes (Cook et al., 1999). On the other hand, the use of more variable genomic regions, such as ORF-K1, is now widely accepted in molecular studies of the epidemiology of HHV-8 for determination of the origin, genetic evolution, transmissibility, and disease associations of this virus (Fouchard et al., 2000; Pérez and Tous, 2017).

The ORF-K1 gene has very high variability. These variations are concentrated in the central region which has two hypervariable regions (VR1 and VR2) (Lacoste et al., 2000a), and with up to 85 % of nucleotide changes in these regions producing amino acid changes (Isaacs et al., 2016; Zong et al., 2007, 1999). Therefore, epidemiologists regard ORF-K1 as a marker of strain diversity and use it to investigate HHV-8 epidemiological spread. This virus is now grouped into six main genotypic clades (A to F) that have distinct distributions among different geographic and ethnic groups, and appear to migrate with the human populations (Hayward, 1999; Isaacs et al., 2016; Liu et al., 2017; Zong et al., 1999).

Evolutionary analysis indicated that genotype B appeared about 100,000 years ago in Africa; genotypes D and E arose about 60,000 years ago on the Pacific Islands and in Amerindian populations, respectively; and genotypes A and C appeared about 35,000 years ago in Eurasia (Marashi et al., 2018; Zong et al., 2002). In agreement with this scenario, genotypes A and C are currently predominant in Europe, the United States, most parts of Asia, and the Middle East, and genotype B is predominant in Africa. The more rare genotypes D, E, and F have only been reported in the Pacific Islands and Taiwan, Amerindian populations of Latin America, and Uganda and Brazil, respectively (Tozetto-Mendoza et al., 2016).

Our phylogenetic analysis of the K1 gene in 24 isolates from Brazil indicated that genomes clustered with genotype A (29.2 %), genotype B (25 %), and genotype C (45.8 %), rather than as a single monophyletic cluster. This suggests that these genotypes were introduced into Brazil at multiple times and from multiple locations (Fig. 1).

Our analysis of the putative origins of HHV-8 genotypes in this study was based on calculations of mean genetic distance. Thus, the putative origins of HHV-8/A isolates were the Tartar ethnic group, Russia, and Ukraine (1.9, 2.2, and 2.2, respectively; Table 5 and Fig. 2). Notably, during the late nineteenth and early twentieth centuries, Brazil received a large influx of immigrants from different countries, including these regions (Spitz et al., 2017), where genotype A is common (Kadyrova et al., 2003; Lacoste et al., 2000b).

The putative origins of HHV-8/B isolates were Congo and Democratic Republic of the Congo (1.7 and 1.9, respectively) (Table 5 and Fig. 2). It is possible that these HHV-8 B strains were brought by African slaves during the colonial period of Brazil (16th century), and that their circulation has increased since then.

Genotype C is common in European countries (England and France) (Cook et al., 1999; Lacoste et al., 2000a)and regions subsequently occupied or colonized by these countries (Australia, Algeria, French Guiana) (Augustyn et al., 2019; Democracy, 2019; Lacoste et al., 2000a; Meng et al., 1999; Sutton et al., 2019). The Brazilian C sequences had the lowest genetic distances for C sequences from Australia, Algeria, England, and French Guiana (2.0, 2.1, 2.1, and 2.1, respectively) (Table 5 and Fig. 2). Therefore, it is likely that the Brazilian C strains were brought into Brazil by immigrants of these countries.

Our findings are thus consistent with the early history of Portuguese colonization in Brazil during the early 16th century, when most residents were African slaves, and the subsequent history when there were waves of migration mainly from Europe, Asia, and the Middle East. Overall, immigrants from more than 60 countries arrived in Brazil before 1930 (Wejsa and Lesser, 2019). Nevertheless, as the samples were obtained from HIV infected individuals the route of transmission could have been more recent through sexual contact on a trip or sex with tourist visiting the country. Further studies including phylogeography analysis could clarify the rout of transmission of HHV-8 in Brazil.

Previous HHV-8 genotyping using the K1 gene has employed different strategies, such as analysis of the complete ORF-K1 region (approximately 870 bp) including both hypervariable regions (VR1 and VR2); separate analysis of the two hypervariable regions to produce two phylogenetic trees; and analysis of the VR1 region only (Pérez and Tous, 2017; Tozetto-Mendoza et al., 2016).Previous molecular epidemiological studies in Brazil have exploited the genetic diversity of HHV-8 ORF-K1 (partial and/or complete K1 gene) and showed a wide spectrum of genotypes circulating in the Brazil, including genotypes A, B, C, E, and F (Biggar et al., 2000; Nascimento et al., 2005; Ramos da Silva et al., 2011; Tozetto-Mendoza et al., 2016).

However, mismatches within the sites of the primers for VR1 and VR2 were observed after analysis of the alignment containing reference sequences of HHV-8. This may have led to biased genotyping if certain primers preferentially amplified one genotype over another. For example, the VR2 assay can lead to underestimation of HHV-8 genotype B (Tozetto-Mendoza et al., 2016).Therefore, phylogenetic analysis based on the whole ORF-K1 sequence is more reliable (Ramos da Silva et al., 2011), because the sites of the primers used for amplification are more conserved (located at both ends of the K1 gene) (Lacoste et al., 2000a; Poole et al., 1999). Thus, we analyzed the complete K1 gene and identified 7 new Brazilian sequences for genotype A, 6 new Brazilian sequences for genotype B, and 11 new Brazilian sequences for genotype C. These new sequences provide new information for future studies of the epidemiology and evolution of HHV-8 in Brazil and elsewhere.

Previous studies proposed that different HHV-8 genotypes may have different pathogenic and tumorigenic potential (Hayward, 1999; Schwartz, 1996). Although we did not study this topic, other studies have confirmed this relationship (Boralevi et al., 1998; Cordiali-Fei et al., 2015; Isaacs et al., 2016; Mancuso et al., 2008; Marshall et al., 2010; Tozetto-Mendoza et al., 2016). However, there are conflicting data in the literature, and the nature of these relationships is still unclear (Kourí et al., 2012; Nascimento et al., 2005; Tornesello et al., 2010). Future studies of the genotypic diversity of HHV-8 may help to clarify this issue.

In this study, we used different samples (serum from patients from Rio de Janeiro, and saliva from patients from São Paulo) for HHV-8 detection. It is well known that HHV-8 can be detected in different body fluids, especially in saliva. Although this detection is reported to be less common in serum (de França et al., 2011), we identified high HHV-8 load average in this fluid ($1.25*10^9$ copies/mL) (Table 3). This can be explained mainly because these patients were immunocompromised. We found that 75 % of these patients from Rio de Janeiro had CD4 + T lymphocyte count < 200 cells/mm^3 blood and HIV load average of $1.69*10^5$ copies/mL (Table 3). In addition, cooperation mechanisms between HIV-1 and HHV-8 may culminate in this HHV-8 spread in the host organism (Lopes et al., 2019).

5. Conclusion

In conclusion, this study allowed us to identify the possible origins

of HHV-8 genotypes A, B, and C in Brazil, and provides new insights about the geographic circulation of HHV-8 genotypes in the world. The HHV-8 ORF-K1 sequences reported here will provide a basis for future studies of the molecular epidemiology and genetic evolution of HHV-8.

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Data availability

The datasets used to support the findings of this study are available from the corresponding author upon request.

CRediT authorship contribution statement

Amanda de Oliveira Lopes: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing - original draft. **Natália Spitz:** Conceptualization, Formal analysis, Methodology, Writing - review & editing. **Katrini Guidolini Martinelli:** Conceptualization, Formal analysis, Methodology, Writing - review & editing. **Anderson Vicente de Paula:** Methodology, Resources. **Ana Luiza de Castro Conde Toscano:** Methodology, Resources. **Paulo Henrique Braz-Silva:** Resources, Writing - review & editing. **Juliana dos Santos Barbosa Netto:** Resources, Writing - review & editing. **Tania Regina Tozetto-Mendoza:** Methodology, Resources, Writing - review & editing. **Vanessa Salete de Paula:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there exist no conflicts of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197828.

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