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Original Article

Characterization of human herpes virus 8 genotypes in Kaposi's sarcoma patients in Tehran, Iran

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Abstract: Kaposi's sarcoma (KS) associated with HHV8 is one of the common connective tissue malignancies especially in immunocompromised patients. The aim of the present study was to determine the common HHV8 genotypes in hospitalized patients in Tehran, Iran. A total of 36 archival paraffin-embedded KS tissue samples of patients with common characterization of KS were collected between 1999 to 2010 from hospitals in Tehran, Iran. After identifying the presence of HHV8 by amplification of its ORF 26 region, the ORF K1 region was amplified, sequenced and used for phylogenetic analysis. Among 30 ORF 26 positive cases, ORF K1 was amplified successfully in 14 cases. Consistent with other studies in Asia, subtype A (9 cases; 64.28%) and subtype C (5 cases; 35.71%) were detected by phylogenetic analysis. This result is in concordance with results from other countries of the region, however the ratio of genotype A to C is higher in our study compared to another study in the country.

Keywords: Kaposi's sarcoma, HHV8, PCR, Tehran

Introduction

Human herpesvirus 8 (HHV8), also known as Kaposi's sarcoma associated herpesvirus (KSHV), is a new member of the subfamily gamma herpesvirinae that has been associated with all forms of KS [1, 2]. Classic KS was first described in 1872 as a rare tumor by the Hungarian dermatologist, Moritz Kaposi. It is characterized as an indolent disease occurring in elderly men of Mediterranean or Jewish descent [3]. The HHV8 also causes body cavity based lymphoma (BCBL) or primary effusion lymphoma (PEL) and multicentric Castlemans disease (MCD) [4]. Four major clinical forms of KS have since been described: 1- AIDS-associated KS, an aggressive form of KS that occurs mainly in homosexual and bisexual men [5], 2- classic KS affects typically elderly men in Mediterranean area, 3- endemic KS, affecting children and young men in central Africa [6], and 4- iatrogenic KS, observed in some patients under immunosuppressive therapy [7]. Sequence analysis demonstrated that HHV8 is closely related to other members of the γ-herpesvirus family, Epstein-Barr virus (EBV) and Herpesvirus saimiri. The 160–170 kb DNA genome of HHV8, with at least 87 open reading frames (ORF) [8], is conserved throughout most of the genome except for the genes at both ends that are considerably variable. ORF K1 is at the left end of the HHV8 genome that encodes a 298 amino acid membrane glycoprotein [9], and contains two hypervariable regions: variable region 1 (VR1) and variable region 2 (VR2). Molecular studies have identified seven main KSHV subtypes (named A-F and Z) on the basis of genetic variability limited to ORF K1 [10]. Subtypes A and C are found in Europe [11], the USA [12], Middle East and Asia [13], Subtypes B and A5 are found mainly in Africa [8, 14] and French Guiana [15, 16], subtypes D was first reported in Taiwan and in some pacific islands [8] and Australia [17], and subtype E has been found among Amerindian

population of the Brazilian [12] and Ecuadorian Amazon regions [18]. Subtype Z has been found in a small cohort of Zambian children [19] and a new subtype F has been identified in the Uganda [20]. In the present study, we investigated the genotypes of KSHV-associated Kaposi's sarcoma in Tehran- Iran, using sequencing of the ORF K1 segments.

Materials and methods

Patient sample

In this cross-sectional study, archival paraffin-embedded KS tissue samples of 36 patients with common characterization of KS by pathological diagnosis were collected between 1999 to 2010 from Loghman, Razi and Shohada hospitals in Tehran, Iran. Samples were transferred to the clinical research department for pathological investigations (**Figure 1**), before molecular testing in the virology department of Pasteur Institute of Iran. In addition, a questionnaire was filled out for each case and clinical data were entered. The ethical considerations of the study were approved by the ethical committee of Pasteur Institute of Iran.

DNA extraction

At least two 20 μm sections (sectioned with razor blade) were obtained from each of 36 formalin fixed paraffin-embedded tissues for DNA extraction. Sections were de-paraffinized with the addition of 100 μl Tween 20 (0.5%) and heating for 90 seconds in Microwave oven at 650 W. Then, the samples were centrifuged for 15 min at 10500 g, placed on ice for 10 min, and treated with 400 μl digestion buffer (50 mM Tris-HCl, 0.05% Tween 20, pH 7.5 and 20 μl proteinase K). The samples were incubated for 3 hours at 55°C and boiled for 10 min in water bath before incubation for 2 hours with 6 μl RNase A (100 μg/ml) at 37°C. After treatment with 300 vs 300 μl phenol/chloroform and centrifugation for 5 min at 12000 g, the upper phase was transferred to a new tube with 1 ml isopropanol and kept at -20°C overnight. After another centrifugation at 12000 g, the precipitated DNA was rinsed with 70% ethanol at room temperature, and centrifuged for 5 min at 12000 g. Finally the pelleted DNA was dissolved in 30 μl distilled water [21]. In our earlier experiments we faced PCR products of almost similar as expected size of K1 bands, which we realized

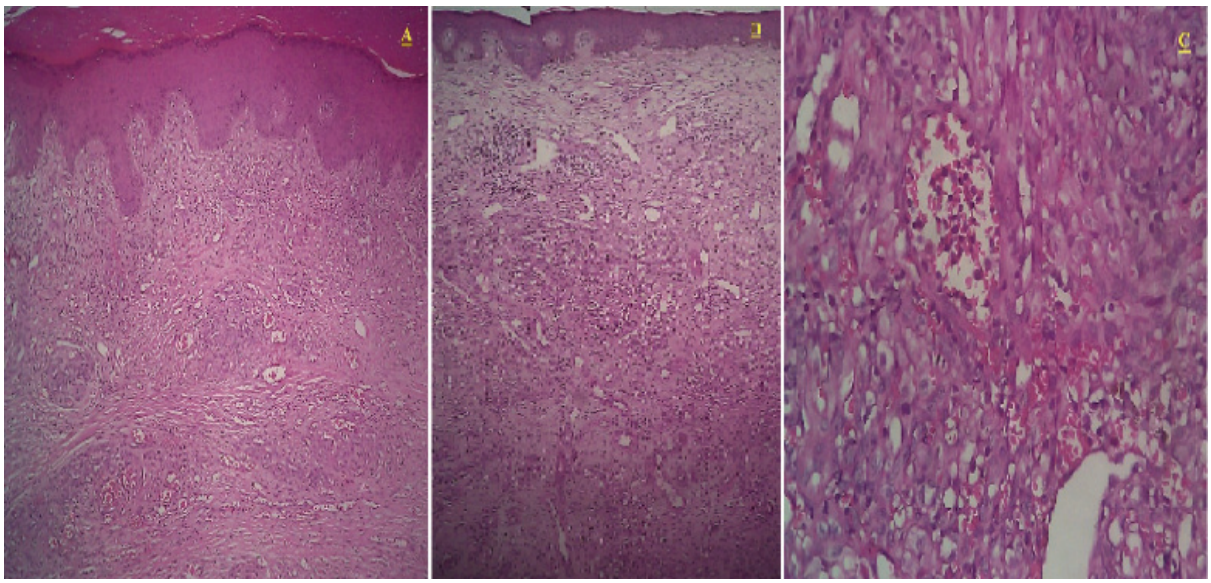

Figure 1. Nodule and Plaques of Kaposi's sarcoma. A. Low power; Reveals normal epidermis, some irregular vascular spaces separating by collagen bundles in association with multifocal cellular aggregates. B. Reveals proliferation of vessels around the preexisting vascular spaces forming promontory sign. Patchy and scattered lymphoid cells and or plasma cells are present. C. High power; Shows dermal vascular proliferation lined by prominent hyperchromatic endothelial cells. A few dilated vascular channel is found at periphery. Extravasated erythrocytes in association with clusters of eosinophilic Hyaline globules are present intra and extracellular aggregates.

to be due to an unusual bacterium (*C. metalidurance*). To avoid such bacterial contaminations, we highly recommend using sterilized cutting instruments for DNA extraction from tissue samples [22].

Polymerase chain reaction

The temperature and cycling conditions of PCR amplification were set up in this study, but K1-N and K1-C primers were adapted from Szalai et al. [23]. The DNA extraction process was validated by β-globin gene amplification using GH20 and PC04 primers [24]. Briefly, a 25μl reaction volume containing 1X PCR Buffer, 200 μM dNTP, 1.5 mM $MgCl_2$, 1.5 U Taq DNA polymerase (CinnaGen, Iran), 100-500 ng DNA, and 0.4 μM of the ORF 26 primers (KS1, KS2, KS3, and KS4), ORF K1-VR1 region primers (K1-C, K1 -N, Nk1-F, and Rev2) and ORF K1-VR2 region primers (K1-C, K1-N, and Forward VR2 primers) were used for nested PCR of ORF 26 and ORF K1-VR1, and semi-nested PCR of VR2 regions. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide, and were visualized under ultraviolet light alongside with the 100-bp DNA ladder (Fermentas, Lithuania). Positive PCR reactions were expected to produce amplicons of 250 bp for β-globin, 138 bp for ORF 26, 400 bp for ORF K1-VR1 region, and 369 bp band for ORF K1-VR2 region, as shown in **Figure 2**. The detailed description of primers and cycling conditions are shown in **Table 1** and **2**.

Phylogenetic analysis of HHV8 genotypes

PCR amplicons were sent to GenFanvaran (Iran, Company) for sequencing. Then, the partial nucleotide sequences obtained from ORF K1 (VR1 and VR2 regions) sequencing were aligned using the ClustalX version 2, and phylogenetic trees were constructed by the neighbor-joining method with 1000 bootstraps using the MEGA 5 software (The Biodesign Institute, USA) [25].

Results

Out of the 36 paraffin-embedded tissue specimens, the 138 bp PCR product of the HHV8 ORF 26 region was detected in 30 (83.33%) by nested PCR amplification. Of the 30 ORF 26 positive cases, the 400 bp amplicon of ORF K1-VR1 region was detected in 9 (30%) by nested PCR. In addition, the 369 bp product of the ORF

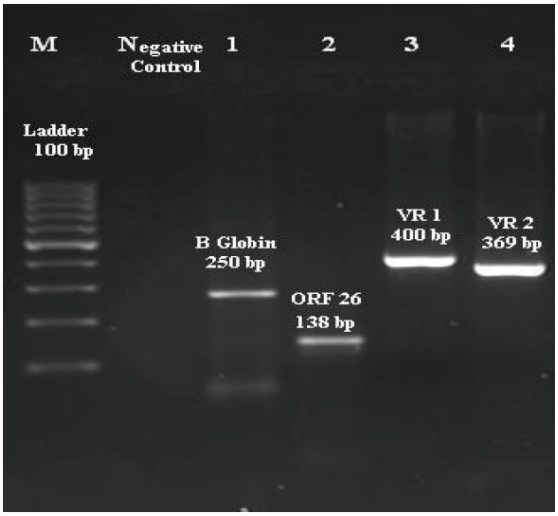

Figure 2. Amplification products of the β-globin and HHV8. Lane1; B Globin, Lane2; ORF26, Lane3; ORF K1- VR1 region, Lane4; ORF K1- VR2 region, respectively. Lane M; Gene Ruler (100 bp plus DNA ladder, Fermentase, SM0323) was used as the molecular size (M) standard.

K1-VR2 was amplified in 14 (46/66%) by semi-nested PCR. We could successfully sequence both ORF K1-VR1 and ORF K1-VR2 segments only in 9 patients Subtype C (5 cases; 35.71%) and subtype A (9 cases; 64.28%) were the identified subtypes in amplifiable DNA samples. Unfortunately, for 22 samples we were not able to attain amplicons of proper quantity and quality for sequencing. Overall, the details of distribution of HHV8 genotypes are shown in **Table 3**. Also, PCR results were consistent with pathological investigation of KSHV patients' lesions (**Figure 1**). The partial sequences of HHV8 isolate were submitted to GenBank database with following accession numbers: ORF K1-VR1: HQ395611- HQ395612- HQ395613- HQ395614- HQ395615- HQ395616- HQ395617- HQ395618- HQ395619, and ORF K1-VR2: HQ395620- HQ395621- HQ395622- HQ395623- HQ395624- HQ395625- HQ395626- HQ395627- HQ395628- HQ395629. Phylogenetic analysis of the nucleotide sequences are shown in **Figure 3** and **Figure 4**.

Discussion

Hayward et al. hypothesized that KSHV is an evolutionary old human virus distributed worldwide along broad ethnic and geographic lines

Table 1. Human β-globin and HHV8 primers.

Primer	Sequence (5′ - 3′)	Amplicon (bp)	Reference
β-globin			
GH20	GAAGAGCCAAGGACAGGTAC		
PC04	CAACTTCATCCACGTTCACC	250	Meshkat et al (24)
ORF 26			
1st round			
KS1	AGC CGA AAG GAT TCC ACC AT TCC		This Study
KS2	GTG TTG TCT ACG TCC AG	233	This Study
2nd round			
KS3	TAT TCT GCA GCA GCT GTT GG		This Study
KS4	TCT ACG TCC AGA CGA TAT GTG C	138	This Study
ORF K1-VR1			
1st round			
K1-N	ATGTTCCTGTATGTTGT		Szalai et al (23)
K1-C	GTACCAATCCACTGGTT	852	Szalai et al (23)
2nd round			
Nk1-F	ACGCCTTACAMGTTGACCTG		This Study
Rev2	TTGACAGGCGAGACGACAGC	400	This Study
ORF K1-VR2			
1st round	ATGTTCCTGTATGTTGT		Szalai et al (23)
K1-N	GTACCAATCCACTGGTT	852	Szalai et al (23)
K1-C			
2nd round	TCGTCTCGCCTGTCAAAT		This Study
VR2 Forward	GTACCAATCCACTGGTT	369	Szalai et al (23)
K1-C			

Table 2. The details of cycling conditions.

	Denaturation		Annealing		Extension		No cycles
	Temperature(c)	Time	Temperature(c)	Time	Temperature(c)	Time	
Human β-globin	95	45″	57	50″	72	1′	40
ORF26- 1st	95	45″	59	45″	72	45″	35
ORF26- 2nd	95	40″	59	40″	72	40″	35
VR1- 1st	95	50″	45	50″	72	55″	35
VR1- 2nd	95	45″	57	45″	72	45″	35
VR2- 1st	95	50″	45	50″	72	55″	35
VR2- 2nd	95	45″	54	45″	72	45″	35

and acts as a marker for ancient human migration [20]. The first expansionary migration was into sub-Saharan Africa starting 100000 years ago (B subgroup), then into South Asia, Australia and the Pacific beginning 60000 years ago (D subgroup), and finally as two major branches into Europe and North Asia (both via the Middle East) about 35000 years ago (A and C subgroups) [12]. In this study, we analyzed HHV8 ORF-K1 polymorphism in 36 Iranian patients with different types of Kaposi's sarcoma. Among them, 14 samples were typable, and all belonged to A and C subtypes. Very limited data were available on HHV8 sero-epidemiology, and just one very recent study on molecular epidemiology of the virus in Iran [26-28]. Subtypes A

Table 3. The information of molecular epidemiology of HHV8 in 36 patients studied

Sex	Age	Underlying Disease	B Globin	ORF26	ORFK1-VR1	ORFK1-VR2	Type	Accession No.
F	82		+	+	+	+	A	HQ395614 (VR1) HQ395627 (VR2)
M	76		+	+	+	+	A	HQ395615 (VR1) HQ395625 (VR2)
M	62		+	+	-	-	-	-
M	44		+	+	-	-	-	-
F	64		+	+	-	-	-	-
M	37	HIV+	+	+	+	+	A	HQ395617 (VR1)
M	60	Under Chemotherapy	+	+	-	-	-	-
M	49		+	-	-	-	-	-
M	57		+	+	-	+	C	HQ395624 (VR2)
M	54		+	+	+	+	C	HQ395618 (VR1) HQ395620 (VR2)
M	63		-	+	-	-	-	-
F	58		+	+	-	-	-	-
M	44	HIV+	+	+	+	+	A	HQ395613 (VR1)
M	49		+	+	-	-	-	-
F	58	Renal transplant	+	+	+	+	A	HQ395611 (VR1) HQ395628 (VR2)
M	71		+	+	+	+	A	HQ395612 (VR1) HQ395626 (VR2)
M*	83		+	+	-	+	A	HQ395623 (VR2)
M	84	Syphilis	-	+	-	-	-	-
M	81		+	+	-	-	-	-
M *	50		+	+	+	+	A	HQ395616 (VR1)
M	84		+	+	-	+	A	HQ395629 (VR2)
M	58		+	+	-	-	-	-
M	68		+	+	+	+	C	HQ395619 (VR1)
M *	68		+	+	-	+	C	HQ395621 (VR2)
M	48		+	+	-	-	-	-
M	65		+	+	-	-	-	-
M	51		+	+	-	+	C	HQ395622 (VR2)
F	68		+	-	-	-	-	-
F	43	Renal transplant	+	+	-	-	-	-
M	70		+	+	-	-	-	-
F	55		+	+	-	-	-	-
M	61		+	-	-	-	-	-
M	58		+	+	-	-	-	-
M	64		-	+	-	-	-	-
F	60		-	+	-	-	-	-
M	75		-	-	-	-	-	-

*- Pathological investigations of these samples are shown in Figure 1.

and C were detected among all the patients based on ORF-K1 polymorphism [28]. It is noteworthy that we found much more subtype A genotypes than this recent study(2/43 vesrsus 9/14), which may show variations in sample collection (mostly because of using non-random methods due to very low prevalence of the disease) have a limiting effect on extrapolating both findings to the general population. Our finding is in accordance with Meng et al. study, based on ORF-K1 polymorphism in Japanese, Kuwaiti and African patients, which suggested A and C subtypes as the most common subtypes in Asia [13]. The genotype analysis of 68 KSHV patients in Japan is also consistent with the result of this study and showed the high rate of

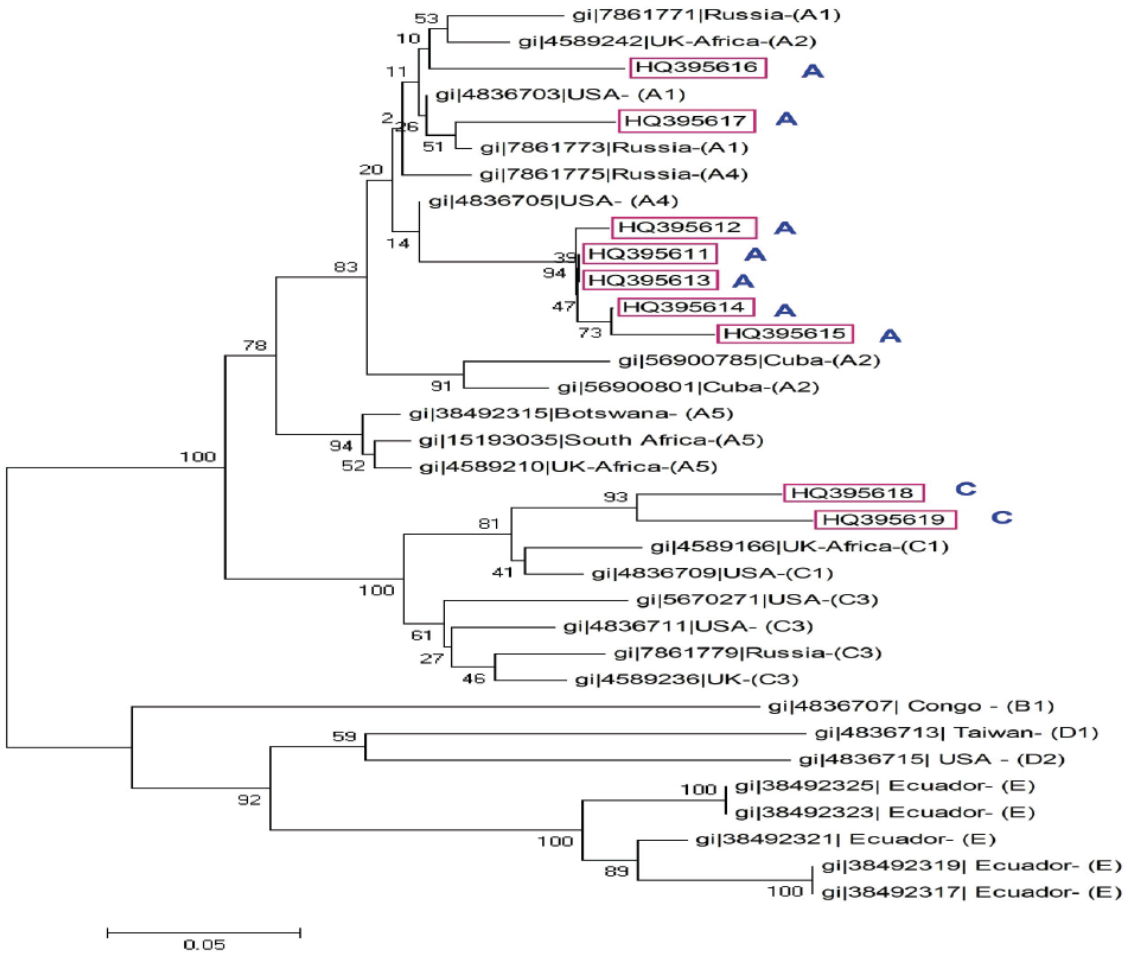

Figure 3. Phylogenetic tree based on nucleotide sequences of ORF K1- VR1 region for Human Herpes Virus 8 isolated from Iran and reference strains. Phylogenetic tree was constructed by the neighbor-joining method using Mega 4. The numbers adjacent to the nodes represent the percentage of bootstrap support (of 1,000 replicates) for each node. The scale bar corresponds to 0.05 substitutions/site. References and GenBank gene identification numbers for the sequences used in ORF K1-VR1 region comparisons; 7861771(Russia), 4589242(UK-Africa), 4836703(USA), 7861773(Russia), 7861775(Russia), 4836705(USA), 56900785(Cuba), 56900801(Cuba), 3849231(Botswana), 15193035(South Africa), 4589210(UK-Africa), 4589166(UK-Africa), 4836709(USA), 5670271(USA), 4836711(USA), 7861779(Russia), 4589236(UK), 4836707(Congo), 4836713(Taiwan), 4836715(USA), 38492325(Ecuador), 38492323(Ecuador), 38492321(Ecuador), 38492319(Ecuador), 38492317(Ecuador).

A and C subtypes [29]. The very recent study of genetic variability of HHV8 in 43 KSHV patients showed that ORFK1-VR1 A/C strains were highly frequent among Iranian patients; most of the sequences (41 of 43) were C subtypes and 2 belonging to A [28]. In addition, subtypes A and C are also prevalent among classic and AIDS-associated Kaposi's sarcoma in Russian patients [11]. Also, findings of Zong et al. [30], Nicholas et al. [31] and DiAlberti et al. [32] revealed the prevalence of A and C subtypes in USA, UK and Italia respectively. In a study by Marchioli et al., biopsies of KS skin lesions accounted for the highest percentage of samples being reactive by HHV8-PCR, with 44 of 48 (91.7%) being positive [33]. This data accompanied with other studies indicate the reliable sensitivity of PCR-based methods for KSHV detection in biological samples [34-38]. The main outcome of this survey will provide useful data for molecular epidemiological studies in Iran and Middle East. It is necessary, to continue the surveillance of cancers such as Kaposi's sarcoma for designing more effective diagnostic

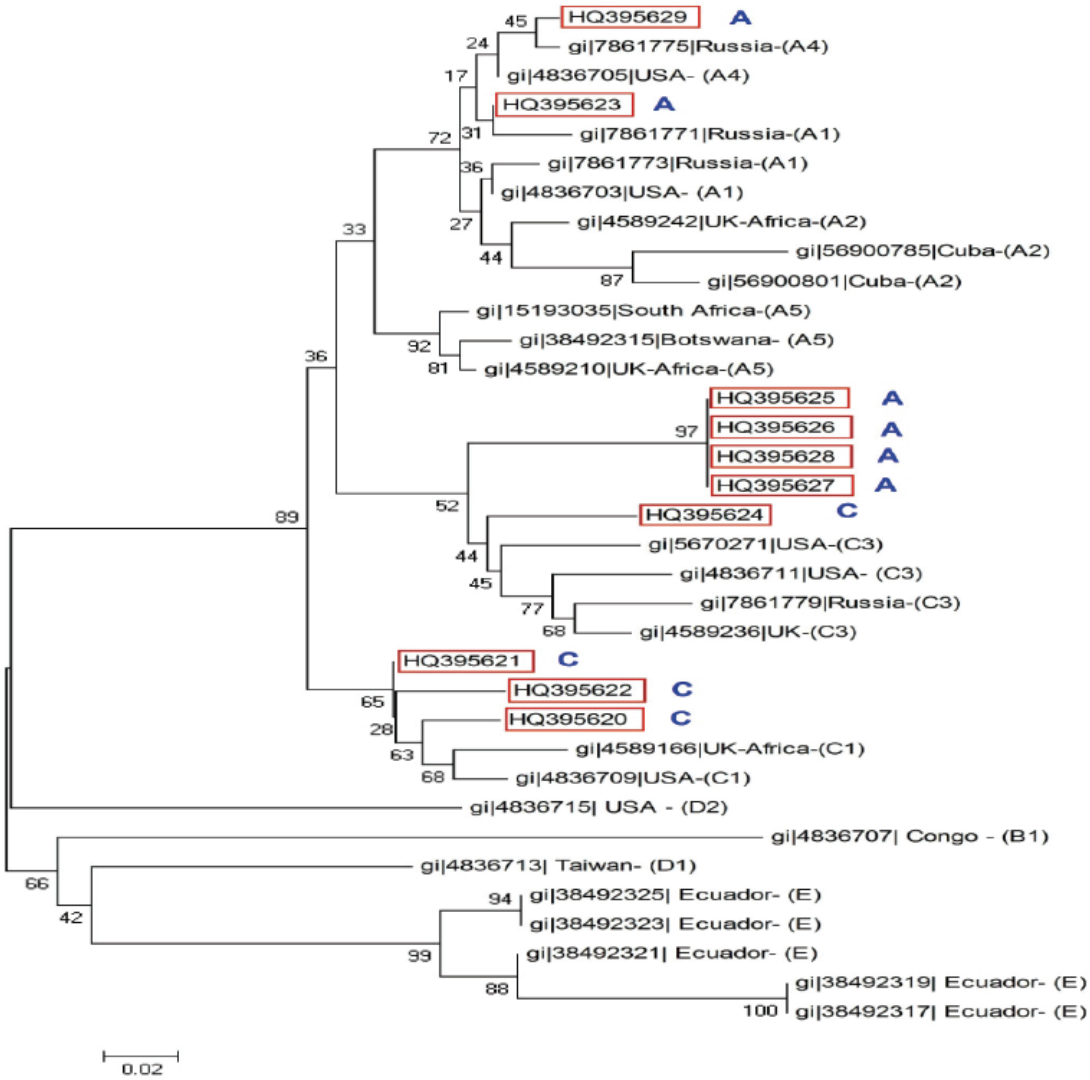

Figure 4. Phylogenetic tree based on nucleotide sequences of ORF K1- VR2 region for Human Herpes Virus 8 isolated from Iran and reference strains. Phylogenetic tree was constructed by the neighbor-joining method using Mega 4. The numbers adjacent to the nodes represent the percentage of bootstrap support (of 1,000 replicates) for each node. The scale bar corresponds to 0.02 substitutions/site. References and GenBank gene identification numbers for the sequences used in ORF K1-VR1 region comparisons; 7861771(Russia), 4589242(UK-Africa), 4836703(USA), 7861773(Russia), 7861775(Russia), 4836705(USA), 56900785(Cuba), 56900801(Cuba), 3849231(Botswana), 15193035(South Africa), 4589210(UK-Africa), 4589166(UK-Africa), 4836709(USA), 5670271(USA), 4836711(USA), 7861779(Russia), 4589236(UK), 4836707(Congo), 4836713(Taiwan), 4836715(USA), 38492325(Ecuador), 38492323(Ecuador), 38492321(Ecuador), 38492319(Ecuador), 38492317(Ecuador).

and treatment procedures.

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