International Journal of Medical Laboratory 2022;9(3):187-197.

Original Article

Distribution of Epstein-Barr Virus and Human Herpesvirus 8 Co-Infections among Human Immunodeficiency Virus-1 Positive Patients

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[DOR: 20.1001.1.24233706.2022.9.3.5.9]
[DOI: 10.18502/ijml.v9i3.10904]

ABSTRACT

Article history
Received: 10 Oct 2021
Accepted: 16 Aug 2022
Available online: 15 Oct 2022

Keywords
Epstein-Barr Virus
HIV
Human Herpesvirus 8
Opportunistic infections; Co-infection

Background and Aims: Among Human Immunodeficiency Virus (HIV)-infected individuals, Epstein-Barr virus (EBV) and Human Herpesvirus (HHV)-8 could cause significant illness as opportunistic infections. The purpose of the present study was to evaluate the prevalence of EBV and HHV-8 in saliva specimens obtained from HIV-1 infected Iranian individuals under the Highly Active Antiviral Therapy (HAART) regimen compared with naïve patients.

Materials and Methods: A cross-sectional study was conducted on 103 HIV-1 positive patients who attended the hospitals affiliated with the Iran University of Medical Sciences, in Tehran, Iran, from 2018 to 2019. Enzyme-linked immunosorbent assay (ELISA) test was performed to evaluate HHV-8 and EBV antibodies. A conventional polymerase chain reaction (PCR) was carried out on saliva samples to detect EBV infection and a nested-PCR assay for HHV-8 infection. SPSS (version 20) was used for statistical analysis.

Results: Patients' mean age ± SD was 43.9 ± 16 (range 18-82 years), and from among 103 participants, 59 (57.3%) were male. The results of PCR showed that HHV-8 infection was found in 19 (18.4%), and EBV infection was found in 61 (59.2%) participants. Also, HHV-8 antibody was detected in 73 (70.9%), and EBV antibody in 97 (94.2%) patients. A significant association was observed between patients under treatment with HAART and HHV-8 DNA or EBV DNA infection in saliva.

Conclusions: HIV-infected patients demonstrated a remarkable rate of EBV and HHV-8 in saliva, which could have a great role in the shedding of viruses. Also, they may contribute to the establishment of further opportunistic infections and devastating complications.

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Introduction

The Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/ AIDS) remains a significant problem worldwide. So far, 770'000 patients worldwide have died from HIV and HIV-related diseases. Viral suppression is accomplished by combining antiretroviral therapy, including a complex of at least three or more Antiretroviral drugs. However, a prolonged lifespan has been observed among HIV-infected patients with the availability of Highly Active Antiviral Therapy (HAART) [1, 2]. However, opportunistic infections (OIs) continue to be the leading cause of mortality among HIV/AIDS-infected patients worldwide.

OIs related to HIV-infected individuals negatively affect the quality of life and accelerate progress towards AIDS in these patients, and, as a result, they drastically decrease the effects of treatment with ART. Following infection with HIV, the host immune system is weakened; consequently, opportunistic infections and malignancies threaten the patient [3]. In these patients, co-infection with opportunistic viruses, such as Epstein-Barr virus (EBV) (or human herpes virus (HHV)-4 and HHV-8 or Kaposi sarcoma-associated herpesvirus (KSHV), occurs [4]. Herpesviruses like EBV are ubiquitous, but KSHV does not have this feature. The prevalence of KSHV in sub-Saharan Africa has been reported to be high (> 50%); however, it is uncommon in Asia, European countries, and the United States (< 10%) [5, 6]. EBV and KSHV are classified as members of the herpesviral family, *gammaherpesvirus* subfamily. *Gammaherpesvirus*es have a great tropism to lymphocytes [7]. Among HIV/AIDS individuals, KSHV infection can lead to Kaposi's sarcoma. This malignancy is caused by cells on the blood vessels or lymph nodes that may spread to other organs [3]. Interestingly, EBV and KSHV can cause latent infection, during which EBV and KSHV can express non-coding RNAs and proteins that cause cellular proliferation, which is essential for their life cycle. Generally, an efficient immune system prevents this mechanism, while in an immunocompromised or suppressed immune system, OIs such as EBV and KSHV could establish malignancies and lymphoproliferative disorders [8]. The EBV involves cancers such as Hodgkin's lymphoma, gastric cancer, nasopharyngeal carcinomas, and Burkitt's lymphoma [9]. In addition, the role of the KSHV has been observed in multicentric Castleman disease, Kaposi sarcoma, and primary effusion lymphoma [6].

Previous studies in the Iranian population have provided limited information on EBV and KSHV among HIV-1-infected individuals [10]. Therefore, the present study was carried out to evaluate the prevalence of EBV and KSHV infections in Iranian HIV-1-positive patients with and without HAART therapy.

Materials and Methods

Study design

A cross-sectional study was conducted to determine the prevalence of EBV and KSHV

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[DOI: 10.18502/ijml.v9i3.10904]

infection in saliva obtained from HIV-infected patients in the Iranian population with and without HAART therapy. Data were collected from patients who underwent treatment at the hospitals affiliated with the Iran University of Medical Sciences, Tehran, Iran, from 2018 to 2019. All participants were asked to sign the informed consent in accordance with the Helsinki declaration. The study was approved by the Ethical Committee of the Research Deputy at Iran University of Medical Sciences, Tehran, Iran (code no: IR.IUMS.FMD.REC. 1398.018).

Study population

Inclusion criteria were being positive for HIV infection based on the data repository, not being at the end stage of the disease (AIDS), and agreeing to participate in the study by signing informed consent. Excluded patients were individuals without detectable HIV viral load and those who did not complete the questionnaire and datasets.

Saliva and serum samples (n=103) were collected from all the patients participating in the study. The laboratory information was collected at admission during the research period. Of all 103 included patients, 59 (57.3%) were male. A professional practitioner filled out the questionnaire for each patient or used the data repository. Also, the patient's demographic characteristics, including gender, age, age category, liver enzyme levels, and CD4 count, were recorded.

Sample preparation

About 3 ml of saliva samples were collected from patients into sterile tubes and stored at −70 °C until use. In addition, 5 ml of whole blood was collected from each patient, and the serum was separated via centrifugation after clotting.

Serologic tests

Anti-EBV IgG Enzyme-Linked Immunosorbent Assay (ELISA) was performed using the Anti-Epstein Barr virus (EBV-VCA) IgG Human ELISA Kit (ab108730, Abcam, Cambridge, United Kingdom), according to kit instructions. The absorbance value of 0.150–1.300 was considered the cut-off point. The positive samples had values more than the cut-off point. Anti-KSHV/HHV8 IgG ELISA testing was accomplished using the human herpes virus type 8 IgG antibody (HHV8-Ab-IgG) ELISA Kit (MBS2800428, San Diego, California, United States), according to the kit instructions. The positive results were reported by OD sample ≥ 0.10 and negative values by OD < 0.10.

DNA extraction

According to the manufacturer's instructions, DNA was extracted from saliva using a DNA extraction kit (QIAamp® DNA Mini Kit, Qiagen, GmbH, Germany). The quantity, quantification, and purity of the extracted DNA (OD 260/280 nm) were determined using the Nano Drop™ 1000 Spectrophotometer by Thermo Fisher Scientific. The extracted genomic DNA was stored at -20 ˚C until use.

Polymerase chain reaction (PCR)

In the present study, the EBV EBNA-3 gene was detected using conventional PCR, and KSHV minor capsid protein (encoded by ORF26) was detected via nested-PCR. In each PCR reaction test, 200-500 µg/ µl of DNA was used. The primers used are shown in Table 1 [11, 12].

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The first round of nested PCR for KSHV was accomplished in a 25µL mixture including 200-500 µg/µl of each extracted DNA, 1.5 U of Taq DNA polymerase, 2.5 µL of 10X PCR Buffer, 10 pM of forward and reverse primers (First round primers), 200 µM mix dNTPs, and 1.5 mM MgCl2 concentration as well as distilled water added to the rest of the volume. Amplification was performed that included pre-denaturation at 95 °C for 5 min (1 cycle), and 35 cycles of denaturation at 95 °C for 40 seconds, annealing at 55 °C or 45 seconds, and extension at 72 °C for 40 seconds, followed by a post extension at 72 °C for 8 min. Then, using the inner primer pair, 200-500 µg/ µl of PCR product of the first amplification was added to the second stage of PCR amplification. In the second round of nested-PCR, the same process was accomplished, as demonstrated in the first round, using the second round primers. The second round was performed using the following protocol: pre-denaturation at 95 °C for 5min (1 cycle), 35 cycles of denaturation at 95 °C for 35 seconds, annealing at 55 °C or 40 seconds, and extension at 72 °C for 35 seconds, followed by a post extension at 72 °C for 8 min. For EBV PCR, a 25 µL mixture including 200-500 µg/ µl of sample or control, 12.5 µl of master mix, 0.5 µl of forward and reverse primers, and 9 µl distilled water were added. Amplification was performed, which included pre-denaturation at 94 °C for 10 min (1 cycle), 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C or 30 seconds, and extension at 72 °C for 30 seconds, followed by a final extension at 72 °C for 5 min.

Using agarose gel electrophoresis, PCR products, and DNA ladder along with positive and negative controls could be visualized.

Quality control

Each ELISA assay for EBV and KSHV qualified by the cut-off standards. In this regard, the EBV cut-off was valued as greater than 1.3 for positive results, and for the KSHV, it was greater than 0.1.

Also, a positive and a negative sample were used for the extraction quality control, then used as a control for PCR. Nucleotide sequencing was used as a confirmatory test for PCR results. One of the positive PCR products from each virus was sequenced; raw data were trimmed by CLC workbench five bioinformatics software and confirmed by Basic Local Alignment Search Tool online software (https://blast.ncbi.nlm.nih.gov).

Statistical analysis

Statistical analyses were performed using SPSS (SPSS Inc, Chicago, IL, USA), version 20. Chi-square and appropriate parametric tests were applied to analyze gender, age category, and patients under treatment with HAART.

Results

The mean age ± SD of the patients was 43.9 ± 16.1 years (range: 18-82 years). Of 103 participants, 59 (57.3%) were male and the age groups were 10-19 years 4.9% (n = 5), 20-29 years 18.4% (n=19), 30-39 years 22.3% (n = 23), 40-49 years 14.6% (n = 15), and 50-89 years 39.8% (n = 41). Different age categories and positive and negative results are shown in Table 2. The mean CD4 count ± SD of all 103 patients was 532.47 ± 212 cells/µL (range 142 -

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995 cells/µL), and the mean aspartate aminotransferase ± SD was 16.50 ± 6.97 IU/L (range 8-35 IU/L) and the mean alanine aminotransferase ± SD was 16.07 ± 6.33 IU/L (range 7-33 IU/L). The present study observed no significant relationship between demographic variables, including age, CD4 count, aspartate aminotransferase, alanine aminotransferase, and gender.

Using the PCR results, KSHV DNA was found in 19 (18.4%) patients and EBV DNA in 61 (59.2%) (Figure 1). Moreover, KSHV antibody and EBV were detected in 73 (70.9%) and 97 (94.2%) patients, respectively (Table 3).

71.8% (74/103) of HIV-infected participants were under treatment with HAART, and 27.2% (28/103) were new cases. The frequency rates of different laboratory tests in HIV-infected patients in these two groups are shown in Table 4.

No statistically significant association was found between gender and KSHV antibody in serum, EBV antibody in serum, KSHV DNA in saliva, and EBV DNA in saliva. Also, no statistically significant association was found between the age category and KSHV antibody, EBV antibody, KSHV DNA, and EBV DNA. In addition, no statistically significant association was observed between patients under treatment with HAART, KSHV, and EBV antibodies. Meanwhile, a significant relationship was found between patients under treatment with HAART, KSHV DNA, and EBV DNA (Table 4).

Table 1. The list of primers used to PCR and Nested-PCR

Virus	Gene	Base pair (bp)	Sequence 5′ to 3′	
EBV	EBNA3C	153	F-AGA AGG GGA GCG TGT GTT GT R-GGC TCG TTT TTG ACG TCG GC	
KSHV	Minor-capsid/ ORF26	233	First round	F-AGC CGA AAG GAT TCC ACC AT R-TCC GTG TTG TCT ACG TCC AG
		138	Second round	F-TAT TCT GCA GCA GCT GTT GG R- TCT ACG TCC AGA CGA TAT GTG C

EBV= Epstein-Barr virus; KSHV= Kaposi sarcoma-associated herpesvirus

Table 2. KSHV DNA, EBV DNA, KSHV antibodies, and EBV antibodies in different age categories

Age categories	KSHV DNA		EBV DNA		KSHV antibody		EBV antibody	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
10-19	2	3	3	2	3	2	5	0
20-29	5	14	10	9	13	5	18	1
30-39	5	18	16	7	17	6	22	1
40-49	3	12	12	3	11	4	14	1
50-89	4	36	20	20	29	10	38	1

KSHV= Kaposi sarcoma-associated herpesvirus; EBV= Epstein-Barr virus;

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Table 3. Demographic parameters for all participants

Parameters		Male	Female	Total	P- Value
No.		59 (57.3)	44 (42.7)	103 (100%)	-
Age		46.5 ± 16.3	40.3 ± 15.3	43.9 ± 16.1	0.250*
CD4 count (cells/µL)		520.7 ± 213.6	548.2 ± 213.4	532.47 ± 212	0.380*
AST (IU/L)		17.3 ± 7.7	15.4 ± 5.8	16.50 ± 6.97	0.745*
ALT (IU/L)		16.8 ± 6.6	15.1 ± 5.9	16.07 ± 6.33	0.799*
PCR KSHV	Positive	11 (18.6%)	8 (18.2%)	19 (18.4%)	0.996
	Negative	48 (81.4%)	35 (79.5%)	83 (80.6%)	
PCR EBV	Positive	33 (55.9%)	28 (63.6%)	61 (59.2%)	0.492
	Negative	25 (42.4%)	16 (36.4%)	41 (39.8%)	
KSHV antibody	Positive	40 (67.8%)	33 (75.0%)	73 (70.9%)	0.464
	Negative	17 (28.8%)	10 (22.7%)	27 (26.2%)	
EBV antibody	Positive	55 (93.2%)	42 (95.5%)	97 (94.2%)	0.468
	Negative	3 (5.1%)	1 (2.3%)	4 (3.9%)	

* Chi-Square

AST= Aspartate aminotransferase; ALT= Alanine aminotransferase; EBV= Epstein-Barr virus; KSHV= Kaposi sarcoma-associated herpesvirus

Table 4. Frequency of different laboratory tests in HIV- infected patients under treatment with HAART or not under HAART treatment

HAART treatment	KSHV DNA		EBV DNA		KSHV antibody		EBV antibody	
Yes	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
	8 (10.8%)	65 (87.8%)	40 (54.1%)	34 (45.9%)	55 (74.3%)	17 (23.0%)	69 (93.2%)	3 (4.1%)
No	11 (39.3%)	17 (60.7%)	21 (75.0%)	6 (21.4%)	18 (64.3%)	9 (32.1%)	27 (96.4%)	1 (3.6%)
P value	0.001		0.031		0.328		0.892	

HAART= Highly active antiretroviral therapy

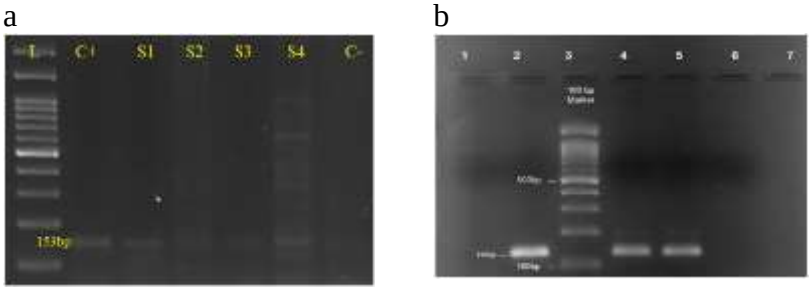

Fig. 1. a) PCR result for EBNA-3C gene of EBV. L: DNA Ladder, C+: Positive control, S1: Positive sample, S2: Negative sample, S3: Positive sample, S4: Positive sample, C-: Negative control. b) PCR result for ORF26 gene of KSHV. 1: Negative control, 2: Positive control, 3: DNA Ladder, 4: Positive sample, 5: Positive sample, 6: Negative sample, 7: Negative sample.

Discussion

According to previous studies, HIV/AIDS is currently a health problem in Iran [13, 14]. So, opportunistic infections such as KSHV and EBV have become important as common HIV/AIDS-related malignancies. In the present study, we examined HIV-1 positive individuals' blood and saliva specimens to understand the prevalence of KSHV and EBV using PCR and ELISA. Generally, we found the following frequency rates: KSHV antibody in serum

70.9%, EBV antibody in serum 94.2%, KSHV DNA in saliva 18.4%, and EBV DNA in saliva 59.2%. Statistically, we have found a significant relationship between being under treatment with HAART and the presence of KSHV and EBV DNA in saliva.

In one study conducted by Hesamizadeh et al. in Iran on 109 HIV-infected individuals, KSHV DNA was detected in plasma and peripheral blood mononuclear cells (PBMC) specimens of 3.6% (n = 4) and 5.5% (n = 6) HIV-infected patients, respectively. The present study detected KSHV DNA in saliva specimens of 18.4% (n = 19) of HIV-infected patients. Our results demonstrated that the prevalence of KSHV DNA in the saliva is higher than that reported by Hesamizadeh K et al. in a similar population. In addition, in our study, the age range of the participants (18-82 years) was not similar to that of Hesamizadeh et al.'s study (2-64 years).

Furthermore, our findings in the current study did not support the results reported by Hesamizadeh K et al. regarding the percentage of patients with the HAART regimen. We had 71.8% (74/103) of the patient under HAART therapy, while Hesamizadeh et al. had only 33% [11]. Thus, the existing differences in the results could be reasonable.

In another study conducted in Iran, KSHV DNA was not reported in HIV-infected patients [10]. In Iran, KSHV DNA in PBMC and plasma samples have been reported to be 5.5% and 3.6%, respectively [11]. In the present investigation, the prevalence of EBV and KSHV indicated that these infections are common in HIV-1 infected individuals in the Iranian population, which is possible because of similar transmission routes of HIV-1 and KSHV infections. However, the results of our study differ from those of some published studies performed in Iran [10, 11].

In samples obtained from 175 patients with nasopharyngeal carcinoma, EBV DNA was observed in 80% of the cases [15]. In Japan, using PCR assay, EBV in saliva was reported to be 48.5% and 15% in individuals with periodontitis and uninfected individuals, respectively [16]. EBV and KSHV DNA in saliva were evaluated in Uganda, too. Shedding of EBV among mothers' saliva was reported to be 72%, and KSHV was 22%; in children's saliva, EBV was 85%, and KSHV was 40%. In saliva, EBV shedding was higher than KSHV shedding [17]. In another study, among HIV-infected individuals, the prevalence rates of EBV DNA, KSHV DNA, Cytomegalovirus DNA, and HSV-1 DNA were reported to be 90%, 57%, 31%, and 16%, respectively [18]. In the Brazilian Amazon region, KSHV DNA in saliva samples was reported to be 23.7% [19], and in Nigeria, the prevalence of HHV8 was 62% in HIV-infected individuals [20]. One study argued that the high prevalence of HHV8 among HIV-1 infected individuals was related to the transmission route of HHV8 by sexual contact [20]. Another study demonstrated that saliva is one of the risk factors associated with HHV infections [18]. Kaposi's sarcoma-associated herpesvirus infection is transmitted through important and different routes, including sexual transmission, saliva, organ transplantation, and blood transfusion [11]. In this study, the main route of KSHV

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[DOI: 10.18502/ijml.v9i3.10904]

transmission among HIV-1 infected patients was sexual contact. The present study's findings confirmed the findings reported by a previous study conducted by Ogoina et al. [20]. Among men who had sex with men (MSM) with HIV infection, oral shedding of KSHV associated with Human Papillomavirus was reported [21]. In a total of 193 MSM with HIV infection, 76.2% EBV DNA in saliva was reported [22]. In saliva specimens obtained from HIV-infected individuals under treatment with antiretrovirals, the prevalence rates of EBV, KSHV, and CMV DNA were reported to be 73%, 24%, and 27%, respectively [23].

In Brazil, among HIV-infected patients, KSHV DNA was reported in 75% of the individuals studied [24] and 40% in saliva samples [25]. In this country, among HIV-infected patients with or without a HAART regimen, HHV infection in saliva was reported to be common [24]. In another study on HIV-infected individuals, HHV-8 DNA was reported in 5 out of 30 (17%) patients [26]. However, the current study's findings are consistent with those reported from various populations related to this infection [24, 26].

The present study's findings seem to be specifically different from those reported in a study conducted in Indonesia [27]. In our study, KSHV antibodies were detected in 73 out of 103 (70.9%) patients, while in Indonesia, this frequency was reported as 7 out of 91 (7.7%). Differences may be explained by rational, geographical, and habitual differences between the two populations, which need further investigation.

Moreover, the current research results do not support those reported in a previous study conducted in Turkey. In our study, KSHV antibodies were reported in 73 out of 103 (70.9%) patients, while in the study from Turkey, KSHV IgG antibodies were detected in 44 out of 173 (25.4%) individuals infected with HIV [28].

The prevalence of EBV and KSHV in participants of the current study reveals that asymptomatic viral shedding in the saliva is a usual phenomenon. One of the Iranian public health goals is to make antiretroviral drugs accessible and free of cost for all HIV-1 individuals [29]. Worldwide access to HAART has decreased mortality from HIV/AIDS-related illnesses, including opportunistic diseases [1]. The present study, similar to other studies, confirms the presumption that HAART has low efficacy on EBV and KSHV shedding in saliva [30]. Nevertheless, our results demonstrated that EBV and KSHV shedding is high in the saliva of HIV-1 infected patients. These findings are consistent with other data that indicated high EBV and KSHV salivary shedding in immunosuppressed HIV-1 infected patients [18, 31, 32].

Generally, EBV has a lifelong persistent and latent infection. Almost half of the children under the age of 5 are infected with EBV, and as many as 90% of adults deal with this virus at some point in their lives. Thus, it is important to consider the patients' age to analyze and evaluate primary infection or reactivation of EBV. Numerous clinical studies have shown that the prevalence of EBV varies (17.5% to 90%) from country to country. In HIV-infected individuals, the shedding rate of EBV is very high [22]. In children, primary EBV infections

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are typically asymptomatic, which usually manifests very mild symptoms or symptoms very similar to other viral infections [33].

Nevertheless, the primary infection of EBV in youths results in approximately half of the patients suffering from infectious mononucleosis. Another study reported that serological methods for identifying EBV infection were less sensitive than other methods, such as EBV real-time PCR [34]. HAART reduces opportunistic EBV infection frequency in HIV-positive patients [35]. In China, the presence of EBV DNA was reported in the saliva of 100% of HIV infected population without HAART [36]. The prevalence of EBV infection can vary in different age ranges. In children, EBV infection prevalence was reported to be more than 50% under the age of 3 and more than 90% at the age of 8 and above [33]. It seems that the results of similar studies are affected by variables, including different groups in different populations and individuals' levels of sexual health.

Commonly, infection with herpesviruses, especially EBV and KSHV, happens in early life, and antibodies can be detected lifelong. Although our results showed that most of our samples had positive antibody results, molecular tests such as PCR, which shows a recent infection, may not be positive in all cases and the same result as IgG antibody results.

A major methodological strength of the present study was the simultaneous assessment of EBV and KSHV DNA/antibodies in saliva specimens obtained from HIV-infected individuals. Nevertheless, our research has some limitations that should be considered before any generalizations. Small sample sizes are one of the limitations of the current study. Also, we did not compare HIV-infected patients and those without HIV infection. Another limitation of the present study was the lack of viral load tests for EBV and KSHV. Simultaneous evaluation of EBV DNA and KSHV DNA in saliva specimens from individuals living with HIV was performed in a few studies. Therefore, we could not make more extensive comparisons due to the limited number of these studies. Moreover, limitations of the salivary-based test include possible cross-reactivity of antibodies with those produced against other viruses. Furthermore, this test is indicated for surveillance and not for early diagnosis.

Conclusion

Our findings showed that the presence of KSHV and EBV DNA in the saliva is high in HIV-infected patients in Iran. It may contribute to further complications in these patients, especially in immunocompromised or immunosuppressed ones. Further studies with a greater sample size and control group should be conducted to obtain more complete results.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors and researchers would like to thank Keyvan laboratory, Tehran, Iran, for providing PCR samples and controls. This research was supported by the Iran University of Medical Sciences (Grant number: 14401).

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[DOI: 10.18502/ijml.v9i3.10904]

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[DOI: 10.18502/ijml.v9i3.10904]