Journal of Techniques, ISSN: 2708-8383, Vol. 5, No. 2, 2023, Pages 141-146
DOI: https://doi.org/10.51173/jt.v5i2.857

JOURNAL OF TECHNIQUES

Journal homepage: http://journal.mtu.edu.iq

RESEARCH ARTICLE - MEDICAL TECHNIQUES

Seroprevalence and Biomarkers Detection of Human Herpes Virus (HHV-8) in Patients with Kaposi Sarcoma

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Article Info.

Article history:

Received
15 August 2022

Accepted
02 November 2022

Publishing
30 June 2023

Abstract

Kaposi's sarcoma (KS) represents the most prevalent malignancy among untreated HIV-positive individuals. Herpesvirus linked with Kaposi's sarcoma (KSHV; also termed as human herpesvirus 8 (HHV8)). In this study, blood samples were collected from 120 individuals, 60 of them had HHV-8 infection with kaposi sarcoma and 60 persons as apparently healthy control. These patients attended Baghdad Teaching Hospital from the period of 15th February 2021 to 15th January 2022. Infections are seen to be more prevalent in the age group of 25-49 years when compared to other groups. The distribution of the biomarkers confirmed that 50, 25 and 80% of the 20 infected patients were positive for Ca19.9, Ca125 and Ca15.3 respectively. The Ca19.9, Ca125, and Ca15.3 biomarkers all produced good results in patients with Human Herpes Virus 8 (HHV-8) infections, leading us to the conclusion that these biomarkers gave favorable results. All of the PCR products showed a positive amplification at 434bp. Phylogenetic analysis confirmed the belongingness to the HHV strain. Further, this could lead to the development of a novel molecular diagnostic tool.

Publisher: Middle Technical University

Keywords: Biomarkers; Human Herpes Virus; (HHV-8); Sarcoma.

1. Introduction

Kaposi sarcoma (KS) is caused by the human Herpesvirus (HHV)–8, in addition to the unique primary effusion lymphoma [1]. Sexual contact can lead to HHV-8 transmission [2] and via nonsexual ways [3]. Although some scientists have discovered viral DNA in sperm as well as normal and malignant prostate tissue [4], other scientists found no viral DNA in the above-mentioned tissues [5]. As HHV-8 is able to cause cancer, is able to be sexually-transmitted, and was found in prostate tissues by certain researchers, then it can be a viable option for infectious agent cofactor [6].

The epithelial cell lines of the 293 human kidney tissue were the most vulnerable [7]. CD19+ B-cells are HHV-8's essential biological repositories [8]. Endothelium has been found to be naturally infected in different cell types [9], monocyte [10], prostate glandular epithelium [11], sensory ganglion cells of the dorsal root [12], as well as spindle cells from Kaposi sarcoma tumors [9].

HHV-8, like other rhadino viruses, may be harmful only in the presence of additional cofactors, e.g. the concurrent HIV infection or the immunocompromised hosts. HHV-8is largely harmless in the natural healthy host [13]. Currently, however, there is really no recognized host else than humans. HHV8, that belonged to genus Rhadinoviridae within subfamily Gammaherpesvirinae , uses B-lymphocytes as cell reservoirs. Thus, many studies have used lymphoid cell lines (such as BC3 and BCBL1) from patients with pleural lymphoma as a viral source for research into the effects of phorbol ester, 12-o-tetradecanoylphorbol-13-acetate, (TPA), that triggers the lysis phase and the later creation of particles of the infectious virus. HHV8 shows no clear cytolyticimpactat the lysis phase and thus, molecular methods are required for its detection (such asamplication of PCR for specific genes like latency factor LANA, or immunostaining of surface antigens K8.1) [14], Human umbilical vein endothelial cells (HUVEC) and the BAC36 strain were used to provide a highly effective cellular model of infection and viral lytic replication. Such a model allows for defining the lytic-latent phase, revealing that viral production begins at 1–2 days and continues to 10–15 days after infections, followed by its entering latent states as episomes bound with cellular DNA-associated histones where it can remain for the complete lifespan of hosts [15]. HHV8 causes considerable changes in cellular biochemistry and physiology during latent infection, involving changes in HHV8 permeability of cells, resistance to toxic medicines, increased resistance to stressful conditions, enhancement of glycolysis as well as higher expressions to the insulin receptor (IR) [16]. All of the acquired properties and conditions may result in oncogenesis and cell transformations like KS and neoplastic inductions of lymphoma cells. Unfortunately, Nucleoside analogue acyclovir and other standard anti-Herpes medications are ineffective against HHV8 during the latent phase and do not prevent HHV8 development during the latent phase

Nomenclature & Symbols			
HHV	Human Herpes Virus	KS	Kaposi Sarcoma
TPA	12-o-Tetradecanoylphorbol-13-Acetate	NS	Non–Significant
SPSS	Statistical Package for the Social Sciences	HS	Highly Significant
U/ml	Units per Milliliter		

because they only reduce viral generation during the lytic phase. Several substances, however, have lately been characterized as having the ability to influence the latency of Herpes virus [17].

If HHV8 is a co-factor in of prostate cancer progression, then HHV8 sero-prevalence in males with disease may be higher than in age-matched controls. In the current study, we determine the seroprevalence and biomarkers detection of Human Herpes Virus (HHV-8) in Iraqi patients with sarcoma. Serum markers' involvement and prognostic importance in uterine carcinosarcomas (CSs).

The purpose of this study was to see if serum CA 125, CA 15-3, in addition to CA 19-9 might be used as prognostic indicators and as markers for illness progression in individuals with CS.

2. Materials and Methods

2.1. Sample collection

Blood samples were taken for this study from 120 subjects, where 60 of them were positive to HHV-8 with sarcoma and 60 of the subjects are healthy volunteers. Blood samples were collected from patients who attended Baghdad Teaching Hospital from the period of 15th February 2021 to 15th January 2022.

2.2. DNA extraction

DNA extraction was performed as described by Guech-Ongey, (2008) with slight modification [18]. In brief, about 0.2ml of blood sample was digested with proteinase K (150µg/ml for 45min at 60°C) in lysis buffer (10mM Tris-HCl pH 7.6, 5mM EDTA, 150mM NaCl, and 1% SDS). The contents are then centrifuged at maximum speed for 10min and the supernatant was collected into a fresh tube. DNA was now purified with phenol and phenol-chloroform-isoamyl alcohol (25: 24: 1) and extracted with ethanol.

2.3. PCR Amplification of HHV8 ORF26

Using oligoprimers and previously published reaction conditions, the HHV8 ORF26 by using nested PCR amplification [19]. Rightward ORF26 was amplified with outer oligonucleotides LGH2574L (5′-CAGAAACAGGGCTAGGTAC-3′) and LGH2575R (5′-GTGCTTGACGATCTGTCC-3′) and with inner oligonucleotides SJF (5′-CTATCTTCAGAGTCTCAG-3′) and SJR (5′-TAGGTACACACAATTTTG-3′). Leftward ORF26 was amplified with outer oligonucleotides LGH1701R (5′-GGATCCCTCTGACAACC-3′) and SJ2R (5′-GCCAAGATTAAATATAGAACTGAG-3′) and inner oligonucleotides LGH1701R and SJ1R (5′-AATATAGAACTGAGACTCTGAAG-3′). A 50µL reaction mixture including 100 to 300ng of template DNA, 5picomol of each primer, 2.5mM MgCl2, 50µM of dNTPs, 5µL of 10x assay buffer and 2.5U of Taq DNA Polymerase was used for the PCR amplification processes (HiMedia). In an ependorf 9700 thermal cycler, DNA was amplified using the following steps: initial 2min denaturation at 94°C, 45 cycles of 55°C for 45sec, 68°C for 1min, 94°C for 15sec and a final 5min elongation at 68°C. Every set of experiments includes a reaction mixture including genomic DNA taken from the NIH 3T3 murine cell line as a negative control. Bidirectional direct sequencing analysis was performed on each HHV8 PCR product.

2.4. HHV8 Real-Time PCR

Each DNA sample's HHV8 viral load was measured using SYBR Green real-time PCR as described by Sudhakar and Raman, (2020) but with slight modifications [20]. Primers studied before [21] were used in the study and ordered from Sigma Aldrich. ORF26LR1FW (5′-GCAGTATCTATCCAAGTG-3′) and ORF26LR2RV (5′-ACAGATCGTCAAGCA-3′) were used and found to produce 434bp product (Table 1). HHV8 viral quantification was done in the Ependorf Real-time PCR Detection System with 200ng of template DNA. About 12.5µL of SYBR Green supermix (Himedia), and 5 pmol each of forward and reverse primers were used and the total volume was made to 25µl. DNA was amplified using the following steps: initial 3min denaturation at 95°C, 50cycles of 55°C annealing for 30sec, 72°C extension for 30sec and a final 5min elongation at 72°C. All the experiments were done in triplicates and the human β-globin gene (FW5′-GAAGAGCCAAGGACAGGTAC-3′, and RV 5′-CAACTTCATCCACGTTCACC-3′) was used as a housekeeping gene to normalize the target DNA.

Table 1. Showing the list of primers used in the study

	Locus	Primer	Sequences (5′-3′)	Base pairs	Size	GC (%)	Tm	Reference
PCR outer	ORF26-3′	LGH2575-R	GTGCTTGACGATCTGTCC	18	620 bp	60	60.34	[22]
		LGH2574-L	CAGAAACAGGGCTAGGTAC	19		58	65.43	
PCR inner	ORF26-3′	SJ-F	CTATCTTCAGAGTCTCAG	18	402 bp	50	56.45	[19]
		SJ-R	TAGGTACACACAATTTTG	18		55	56.3	
PCR outer	ORF26-5′	LGH1701R	GGATCCCTCTGACAACC	17	589bp	50	52.3	
		SJ-R2	GCCAAGATTAAATATAGAACTG	22		56	52	[19]
PCR inner	ORF26-5′	LGH1701R	GGATCCCTCTGACAACC	17	579bp	58	61	
		SJ-R1	AATATAGAACTGAGACTCTGAAG	22		60	61.23	[19]

Real time PCR	ORF26-3′	ORF26LR1F1	GCAGTATCTATCCAAGTG	18	434bp	55	56.6	[21]
		ORF26LR2R2	ACAGATCGTCAAGCA	15		55	56.4	

2.5. Sequencing and phylogenetic analysis

Eurofins Genomics (Bangalore, India) used fluorescent dye terminator technology and ABI 3730 DNA sequencers to perform bidirectional direct sequencing on the obtained PCR products (Applied BioSystems, Foster City, CA). Sequences obtained in FASTA format were studied by clustal W to depict the phylogenetic relation to the reference sequences. Reference sequences used in the study were DQ984689.1 and DQ984768.1 (HHV8 ORF26 subtype).

2.6. Statistical analysis

The SPSS-20 software program (Faculty version) was used for statistical analysis of data, involving the t-test. ($P<0.05$) value was regarded as statistically significant.

3. Results

3.1. Seroprevalance

Table 2 showed the infection distribution according to gender. The number and percentage of males are 33 (55.0%), and females were 27(45.0%). There was no significant difference obtained in the number based on gender as observed (chi-square 0.03; P=0.8).

Table 2. Distribution of patients group according to the gender

Gender		Studied groups	
		Patient	Control
Male	N (%)	33 (55)	30 (49.20)
Female	N (%)	27 (45)	30 (50.8)
Total	N	60 (100)	60 (100)

From our findings, we could see age group 25-49 was more prone for infections with 45% than other groups ($P<0.05$). However, there is no significant effect seen between the age groups and infection (P =0.25). We found 20, 45, 16.7 and 18.3% of infected among the age groups 5-24, 25-49, 50-64 and >65 respectively. These findings are compared to control groups (Table 3), (chi square 3.12, P=0.25).

Table 3. Distribution of study group according to age group (Years)

Age group/ Years		Studied groups		Total
		Patient	Control	
(5-24) yrs	N (%)	12 (20)	18 (30)	30 (50)
(25-49) yrs	N (%)	27 (45)	27 (45)	54 (90)
(50-64) yrs	N (%)	10 (16.7)	10 (16.7)	20 (33.4)
>65	N (%)	11 (18.3)	5 (8.3)	16 (26.6)
Total	N (%)	60 (50)	60 (50)	120 (100)

We found Ca19.9 biomarker was significantly observed within the subjects when compared to control ($P<0.05$). Table 4 showed the distribution of Ca19.9 biomarker in patients with HHV-8 infections wherein we could see that 10(50.0%) of the 20 infected patients were positive with Ca19.9 (P-value =0.03).

Table 4. Prevalence of Ca19.9 biomarker according to the HHV-8 infection

HHV-8		Ca19.9		Total
		0-37 U/ml	>37 U/ml	
<1	N (%)	40 (100)	0	40 (100)
>1	N (%)	10 (50)	10 (50)	20 (100)
Total	N (%)	50 (83.3)	10 (16.7)	60 (100)

We found no significance in the expression of Ca19.9 biomarker within the subjects when compared to control (P=0.3). Table 5 showed the distribution Ca125 biomarker in patients with HHV-8 infections explained that 5(25%) of 20 infected patients were positive with Ca125, P-value =0.3.

Table 5. Prevalence of Ca125 biomarker according to the HHV-8 infection

HHV-8		Ca125		Total
		0-35 U/ml	>35 U/ml	
<1	N (%)	38 (95)	2 (5)	40 (100)
>1	N (%)	15 (75)	5 (25)	20 (100)
Total	N (%)	53 (83.3)	7 (11.7)	60 (100)

We found Ca15.3 biomarker was significantly observed within the subjects when compared to control (P=0.001). Table (6) showed the distribution Ca15.3 biomarker in patients with HHV-8 infections explained that 16(80.0%) of 20 infected patients were positive with Ca15.3, P-value =0.001.

Table 6. Prevalence of Ca15.3 biomarker according to the HHV-8 infections

HHV-8		Ca15.3		Total
		0-37 U/ml	>37 U/ml	
<1	N (%)	37 (97.3)	1 (2.7)	38 (100)
>1	N (%)	4 (20)	16 (80)	20 (100)
Total	N (%)	41 (70.6)	32 (29.4)	58 (100)

All of the 60 samples showed positive amplification for the HHV8 ORF26 gene. A PCR product of 434bp was seen among all the samples (n=60 positive) which was sequenced. Only 20 samples were sequenced and the results were analyzed using MEGA 7. The phylogenetic tree (Fig. 1) showed a close relation between the samples and all the samples were confirmed to be HHV8 irrespective of their regions.

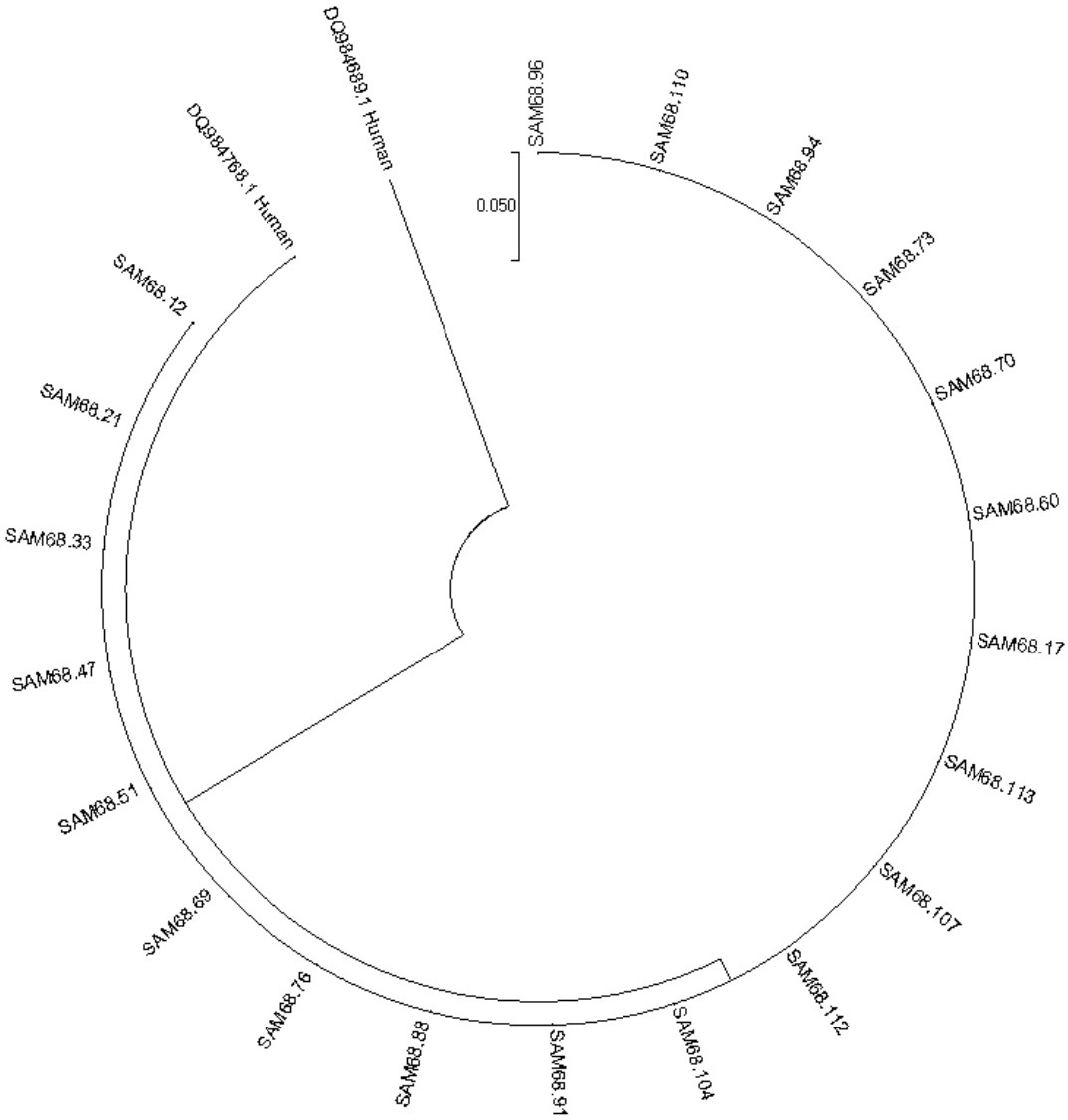

Fig. 1. Showing the evolutionary tree of the DNA sequences using the Neighbor-Joining method [23]. Maximum Composite Likelihood was used to determine distances between 22 different nucleotide sequences. All the gaps and missing data were removed and the final dataset contained about 961 positions. Phylogenetic analyses were done with MEGA7 [24]

4. Discussion

In humans, herpesvirus (HHV8) causes Kaposi sarcoma (KS) and therefore a rarer primary effusion lymphoma. The distribution of HHV8 infections according to the gender was illustrated the male was 33 (55.0%) and the female 27(45.0%).These findings agreed with (Tembo et

al., 2017) who reported that the mostly males (64.4%, 56/84) than females (33.3%, 28/84) [25]. These findings agreed with Mamimandjiami et al., 2021 who found that males more than females were infected due to human herpes virus (HHV8) [26]. Also Chavoshpour-Mamaghani et al., (2021) reported that male was higher significant affected than female in humans, herpes virus (HHV8) [27]. The prevalence of HHV-8 infections, the ages between (5-24) yrs were 12(36.0%) compared with control group 18(64.0%), the ages (25-49) yrs 27(50.0%). Nzivo et al., (2019) were discovered that in the age categories 25-29, 74 (27.6 %) 18-24, 55 (20.5 %) 30-34, and 52 (19.4 %) were above 35 [28]. Shakir et al., (2018) proved that no significant differences among ages with humans, herpes virus (HHV8), $P<0.05$. In humans, herpes virus (HHV8) is one of the most dangerous types that may lead to death. Recently, many cases of death have been recorded when it hits organs in the human body such as the heart and liver, and this leads to death because of its transmission to those organs without symptoms. This virus weakens the immune system and causes great damage, and may help in the programmed killing of the cells of those organs, as it is a silent killer [29]. The distribution Ca19.9 biomarkers in patients with HHV-8 infections explained that 10(50.0%) of infected patients were positive with Ca19.9. CA 19.9 rise is not associated with neoplastic illness. Ca19.9 marker levels are increased in the sera of patients with pancreatic cancer, as it is an indication of tumor occurrence. These results are used with histological and PET SCAN examinations, as they may be an important guide for the physician in the initial diagnosis. And the high levels of Ca19.9 is a marker not only for determining the incidence of pancreatic tumors, but perhaps there are neighboring organs that have been invaded by these tumors, such as the liver, so this indicator is of great importance in determining these cancers [30]. Lyu et al., (2021) has been proven a high percentage in levels of CA19.9 for patients with pancreatic tumors and that this indicator is of great importance in determining glandular tumors, especially pancreatic tumors. While in the benign tumors of the pancreas, there were average levels in the proportions of measurements of CA19.9 [31]. Poruk et al., (2013) explained there is a high accuracy and sensitivity in using the CI 19.9 marker in determining the incidence of pancreatic cancer compared to benign tumors [32]. The distribution Ca125 biomarker in patients with HHV8 infections explained that 5(25.0%) of infected patients were positive with Ca125. Funston et al., (2020) explained that the CA125 values are provided that correspond to the nearest integer probability of 3%. The probabilities, which correspond to a CA125 level of 35 U/ml, are also indicated [33]. Also Edula et al., (2018) agreed with the report of Funston et al., (2020) [34]. The distribution Ca15.3 biomarkers in patients with HHV8 infections explained that 16(80.0%) of infected patients were positive with Ca15.3. These results agreed with (Shakir et al., 2018) who proved in terms of the tumor marker CA15-3, one patient (7.1%) tested positive for HHV8 antibodies. In terms of the PR, ER (expression of estrogen receptor (ER), progesterone receptor (PR)), and HER2/neu, 9 of the 45 patients were tested, and no instances positively tested for HHV8 IgG antibodies. Statistically, the discrepancies between the HHV8 IgG antibodies and the various tumor markers were significant [29]. But Chu et al., (2016) disagreed with present study and reported no significant effect of CA15-3 in HHV8 infection. High levels of CA15-3 in the sera of patients suspected of being blind, because breast cancer is the most common because this marker is one of the indicators of the occurrence of tumors in the human body. In addition, the measurement of this indicator can be useful for those who suffer from common injuries, especially women; chest tumors may be associated with other sites of cancer cervical tumors [35]. The existence of HHV8 DNA was verified by running nested-PCR product on a 1.5 percent agarose gel with a product size of 233bp. Tembo et al., (2017), his research stated that genes are determined with the extracted DNA was utilized to detect the HHV8 sequences. The ORF26 gene KS-1 (5'-AGCCGAAAGATTCCACCAT-3') and KS-2 (5'-TCCGTGTTGTCTACGTCCAG-5'), KS-4 (5'-CGAATCCAACGGATTTGACCTC-3') and KS-5 (5'-CCCATAAATGACACATTGGTGGTA-3') primers were used to amplify the HHV-8 genomes [25]. Ramezani et al., (2016) proved that similar product with primers were used with HIV infection [36].

5. Conclusions

We concluded that Ca19.9 biomarkers, Ca 125 biomarker and Ca 15.3 biomarker gave positive results in patients due to Human Herpes Virus 8 (HHV-8) infections. In conclusion, HHV8 DNA sequences are detected in a substantial proportion of HIV-positive patients. The potential of the amplified gene segment as a molecular diagnostic tool has to be investigated in greater depth.

Acknowledgement

We would like to express our gratitude and recognize Dr. Jaleel Najah Samanje's help in every way possible in providing statistical analysis tools. Also, we want to thank all of the volunteer participants who supplied their blood samples so graciously.

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