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RESEARCH ARTICLE

Molecular Detection and Genotyping of Human Herpes Virus 8 in a sample of Iraqi Blood Donors

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ABSTRACT:
Human herpes virus-8 (HHV-8) infection has increased recently in Arabic countries. HHV-8 in healthy persons does not necessarily cause life-threatening infection, and however, it causes a more severe infection among immunocompromised patients. The distribution of HHV-8 genotypes varies according to ethnicity and depends on the geographic region prior rapid development of global travel. A cross sectional prospective study included a hundred healthy blood donor samples with a mean age of (36.60±10.381), 81% were positive for molecular detection of HHV-8 DNA. PCR results for HHV-8 were strongly related with risk factors such as the number of sexual relations, previous surgeries, blood transfusion, dental operation, and the number of blood donations. In this study, genotypes (A, B, C and D) were detected, largely associated with blood donors residences and distributed to areas of Iraq through a map. Genotype A comprised 28 (34.6%) of blood donors and for genotype C it was 16 (19.8%) and both genotypes were found to be the predominant genotypes, followed by genotype B of 7 (8.7%) and D of 2 (2.5%), the latter is included into Mixed genotypes of 8 (9.9%), whereas, 22 (27.2%) were undetermined genotypes. Efforts should focus on these findings, which may indicate that Iraq is an endemic region of HHV-8 infection.

KEYWORDS: HHV-8, KSHV, Genotypes, Blood donors.

INTRODUCTION:
Human Herpes Virus-8 (HHV-8) is a gamma herpesvirus that is always associated with Kaposi's Sarcoma (KS), hence known as Kaposi's Sarcoma Associated Herpesvirus (KSHV), this virus mainly associated with three types of malignancies that include Kaposi's Sarcoma, primary effusion lymphoma, and multicentric Castleman's disease[1]. Also it is responsible of all clinic-epidemiological forms of KS and lymphoproliferative disorders[2]. KHSV infection is necessary, along with other co-factors not yet clearly identified for the development of Kaposi's Sarcoma as immune dysfunction, sex and genetic backgrounds[3]. The exact route of HHV-8 spread is still under discussion.

Though, previous reports specified that HHV-8 can be transmitted via saliva, sexual and non-sexual routes, blood or blood components and during organ transplantation[4]. The spread of this virus has been recorded in most parts of the world and in the previous years in our Arabic world and in neighboring countries, such as in Saudi Arabia, the seroprevalence was 1.7% in healthy individuals and 18% of recipients of renal transplantation[5]. In Jordan, an elevated percentage of (41%) was found in patients with multiple myeloma compared with absence of virus in all patients with other hematological malignant diseases as well as in normal healthy persons[6]. While among the Iranian population, the 2% seroprevalence of HHV-8 was reported among blood donors[7]. However, 5.3% were reported among blood donors in Turkey[8]. Genotyping of KSHV was based on the sequencing of viral region open reading frame K1 that encoding for a transmembrane protein and containing two hypervariable regions (VR1 and VR2). This has allowed the identification of seven strains including the four main KHSV subtypes (A, B, C and

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D)[9], which are distributed differently between the world. In the Mediterranean region, Kaposi's Sarcoma has a particularly high prevalence rate in HIV patients. Iraq is located close to the Mediterranean region where the spread of HIV is increasing. HHV-8 during latent stage does not show the clinical picture but at some stages, KS is clinically similar to other vascular or skin malignancies for that the detection of viral DNA by PCR method is the golden key to confirm the infection and can help in the identification of non-diagnostic cases. Many studies have been published on the treatment of KSHV[1,10]. Also the virus has been detected in breast cancer Iraqi women[11]. Since the discovery of KSHV there have been limited studies conducted on HHV-8 genotyping in the Middle East. Furthermore; the prevalence of HHV-8 genotypes is different in various regions of the world, in different races and in different clinical forms and stages of the diseases. Recently genotype A and C was detected in Iran[12]. It remains unclear whether the different genotypes are related to age, geographical distribution, race and diverse rates of disease progression corresponding with the immune status, antibody titers and viral load and other possible alternate markers on the outcome of KS that are still under debate.

MATERIAL AND METHODS:

Study group:

This is a cross sectional prospective study included a hundred blood samples collected from apparently healthy blood donors who came to the Iraqi National Center for Blood Transfusion in Baghdad city. This study was conducted at University of Baghdad/ College of medicine/Department of Microbiology. The study extended from February 2019 to April 2020.

Ethical consideration:

The ethical approval of the study was taken from the Ethical Committee in the Department of Microbiology and from the Council of Collage of Medicine/ University of Baghdad/Iraq.

All patients received a written and verbal data sheet explaining the purpose of the study. The written consent signed by each individual participating in the study was obtained prior to a direct interview to record their information and history.

Sample collection:

Ten ml of fresh blood was aspirated from cubital fossa veins using 10ml syringe, some of the sample collected directly from blood pouch using a 10ml vacuum tube. The blood was placed in two EDTA tubes. The blood was left to clot and immediately frozen at -20 that were stored for later DNA extraction, genomic detection and genotyping of HHV-8.

Viral DNA extraction:

Virus DNA extraction was provided for 100 whole blood samples using two (QIAamp DNA blood Mini Kit Cat no/ID:51104 ;for 50 samples by QIAamp® genomic DNA kits). The protocol was adopted in accordance with manufacturer's instructions and to the laboratory practicing.

Viral DNA purity and concentration were measured using nucleic acid measuring instrument (nanodrop) from (ACT GENE, NAS -99), quality tested by amplifying the human housekeeping gene β-globin. All samples were suitable for viral DNA amplification.

Primers design and PCR protocols:

Primer design of viral DNA according to the open reading frame of the K1 Gene taken from Meng *et al.*[13]. Primers used for genotyping of type A, B, C, and D was designated and optimized specifically for this study according to Gene bank database. Primer for human beta globin amplification was standard PCO3, PCO4 as illustrated in (Table- 1). While amplification protocols after optimization of each primer have been used to amplify specific DNA band. The same condition was used in amplifying K1 gene, type B and C but with different annealing temperature. Others also have been improved to amplify type A and D with different annealing temperature with 48 °C (for the K1, B and C) and 54°C (for A and D) detection.

Table- 1: Primers design for K1 Gene detection and Genotyping of KSHV

Gene	Primer	Nucleotide position	Reference
K 1 (NC_009333.1)	5´-GGCCCTTGTGTAAACCTGT-3´ 5´-AGTATCCGACCTCATAAAATG-3´	51-69 1031-1051	Meng *et al* [13]
Type A (AF133038.1)	5´-ATACTCGGCTTTTCCGACCG-3´ 5´-GCTCTGTCGATGCCCAGATT-3´	265-284 359-340	This study
Type B (AF133040.1)	5´- CTGGAGTGATTTCCACGCCT-3´ 5´- AGTCCCGTTGCAATACCAGG-3´	190-209 269-250	This study
Type C (AF133041.1)	5´- CAACGCCTTACACGTTGACC-3´ 5´- CATGCGTCAGTCGGAAAAGC-3´	202-221 291-272	This study
Type D (EF589758.1)	5´-GGCCCTTGTGTAAACCTGT-3´ 5´-AGTATCCGACCTCATAAAATG-3´	159-178 239-220	This study
Human Beta globin/ PCO3	5´-ACACAACTGTGTTCACTAGC-3´ 5´-CAACTTCATCCACGTTCACC -3´	Product size 110	

STATISTICAL METHODS:

Statistical Analysis implemented with statistics for Windows Software System - IBM SPSS, Version 24.0. (2016), program was used to detect the effect of difference factors in study parameters. To identify statistically significant associations, the following tests were used appropriately; One-way ANOVA analysis of variance was used to asses the differences between means of three groups. Chi-square test of independence with Fisher exact test was used to compare between percentages. In this study each tests were two–sided significant; P value (0.05 and 0.01 probability).

RESULTS:

Demographic distribution of blood samples:

In this study, blood samples were randomly taken from blood donors, 92 were males and 8 were females with a male to female ratio of 11.5:1 and their ages ranged from 18-60 years with mean of (36.60±10.381) years.

Viral DNA and Genotypes detection:

All blood samples are independently amplified with negative control and human housekeeping gene β-globin using PreMix from Bioneer AccuPower®. PCR products resolved and detected by 1% agarose gel electrophoresis with ethidium bromide staining. Of a hundred DNA samples an 81% were positive for HHV-8 DNA. All HHV-8 positive DNA samples that were subjected to genotyping by amplifying specific primers belong to a detailed sequence of every four major genotypes (A, B, C and D); Figure -1. The result showed that genotype A comprised 28(34.6%) and for genotype C it was 16 (19.8%), it was found that both genotypes were predominant, followed by B of 7(8.6%) and D of 2 (2.5%), the latter genotype was included into mixed genotypes that accounted 8(9.9%) other viral DNA samples that were not identified in main genotypes were categorized as "undetermined" and consisted of 22(27.2%).

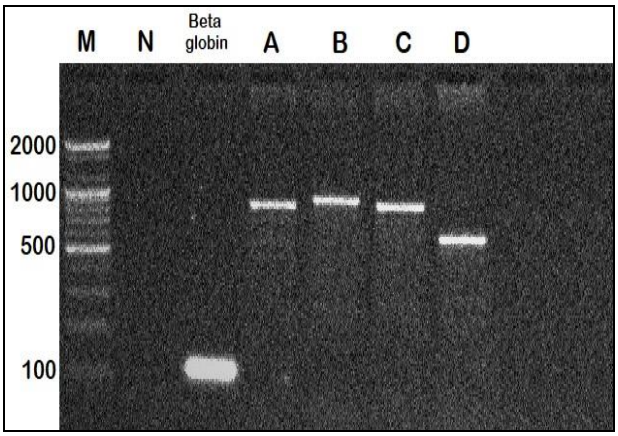


Figure-1: Amplification products of HHV-8 genotypes: Lane M; Gene Ruler (100bp DNA ladder)). Lane N; negative control (deionized water). Lane Human Beta globin; housekeeping gene of 110 bp. Lane A; genotype A at 945 pb. Lane B; genotype B at 960 bp. Lane C; genotype C at 939 pb. Lane D; genotype D at 573 pb.

Risk factors associated with HHV-8:

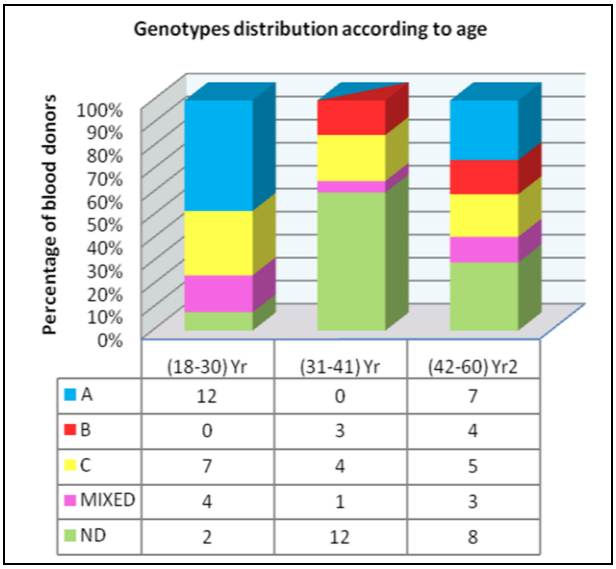
In this study, viral PCR results were significantly associated with many risk factors among blood donors such as sexual relations of about 81%. Other important risk factors were also highly relevant to our results such as surgical and dental operations, blood transfusion, cupping, tattooing, and smoking as shown in (Table- 2).

The study group ranged in age from 18-60 years with mean (36.60 ± 10.381) years. Genotypes identification was significantly related to age groups (P<0.05). The highest frequency of predominant Genotypes (A & C) was found in the age group (18-30) years (Figure -2). Genotyping results were also significantly related to residence of blood donors (P = 0.025). Most of the areas recorded in our study were from Baghdad Al-jadeeda city of 11%, 7% in Al-Sadr City and 6% distributed equally in people resident in Al-Shaab, Al-Bayaa, Al-Hussainiya and Al-Shula as distributed on Baghdad city map (Figure- 3).

Table-2: Risk factors associated with HHV-8 PCR results of Iraqi blood donors.

HHV-8 PCR results				
Risk factors	**Positive No. (%)**	**Negative No. (%)**	***Total NO.**	**P- value**
dental operation	64 (79.0)	17 (21.0)	81	P = 0.042
surgical operation	31 (91.2)	3 (8.8)	34	
blood transfusion	17 (100.0)	0 (0.00)	17	
Smoking	53 (89.8)	6 (10.2)	59	P = 0.011
Cupping	33 (63.5)	19 (36.5)	52	
Tattoo	13 (81.3)	3 (18.8)	16	
Drug used	4 (80.0)	1 (20.0)	5	
Sexual relations	81(81)	19(19)	100	P = 0.002

*From total 100 blood donors, each donor may have many risk factors.



	(18-30) Yr	(31-41) Yr	(42-60) Yr2
A	12	0	7
B	0	3	4
C	7	4	5
MIXED	4	1	3
ND	2	12	8

ANOVA (F = 3.259, df = 2, P = 0.044).

Figure-2: Distribution of HHV-8 Genotypes according to age groups.

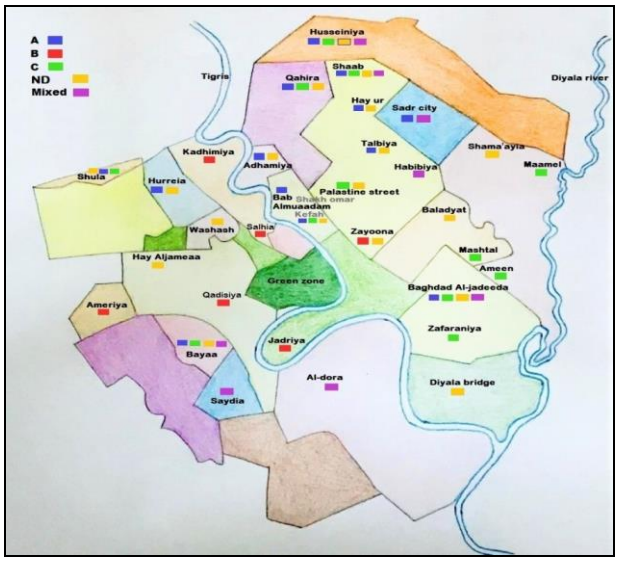


Figure-3: Distribution of HHV-8 Genotypes among Iraqi blood donors in Baghdad city, blue square for type A, red square for type B, green square for type C, yellow square for type D, Purple square for Mixed types and orange square for other genotypes not-determined in this study.

DISCUSSION:

Since the discovery of HHV-8 of more than twenty years ago and until recently huge efforts were spent to prove the potential transmission risk of HHV-8 through blood transfusion. According to increasing demands in our country for blood transfusion for those with post war trauma and for various medical or surgical conditions, many routine tests were applied to blood donors around the world to ensure safe blood is delivered to immunologically susceptible patients[14-16] and effective teaching programs on blood transfusion were adopted[17-22]. So far, HHV-8 tests are not routinely recommended except in an endemic area as several facts point to a possible role of HHV-8 in the pathogenesis of many diseases. More importantly, the majority of blood recipients suffer from critical immune disorders that favor HHV-8 activation. The decision to test blood donors came from the ascending spread of HHV-8 in the Arab world and in the surrounding countries, according to our knowledge there was no such proven study or statistic in Iraq for further interpretation apart from recent study by Sharquie and his co-workers who warned against increasing KS in Iraq most of them were not HIV related explaining the multiple factors played a role in the emergence of KS[10].

In this study, the mean concentration ± SD of the extracted DNA was (33.354±18.3297) ng/ml, with range of (104.5). Another study observed that the type and methods of technical DNA extraction directly affect the detection of viral DNA also on the evaluation of DNA samples with beta globin gene[23]. The extracted HHV-8 DNA had a high purity as confirmed by a Nanodrop device with an O.D. reading ratio of 260/280 nm. Suitable for measuring DNA purity above > 1.7[24]. The result of a housekeeping gene were 100% positive for 100 DNA samples, this result may indicate the importance of using a specific quality of the viral DNA extraction kit on viral detection results, as stated by Demeke and Jenkins study who cited the importance of DNA extraction and its impact on the results[25].

Based on the alignment of each sequence from NCBI GenBank database, several primers have been designed to achieve specific results and in order to optimize our PCR protocol, both forward and reverse primers in the same GC and TM contents must be designed to be easily used afterwards for gradient optimization in order to obtain a specific annealing temperature. ORF K1 was selected to detect KSHV in blood donors; the wide use of high variable genomic regions, such as ORF-K1, is currently accepted in molecular studies of the epidemiology of HHV-8 to determine the viral origin, evolution of viral genetic, transmissibility, and viral associated diseases[26]. According to different studies, high frequency for HHV-8 DNA detection has rarely been recorded among blood donors, and most studies adopted serological analyses. According to a previous study in Tanzania, 20% of blood donors were identified with HHV-8 DNA from their serum samples[27]. HHV-8 not ubiquitous around the world, and the apparent disparity in virus epidemiology was holding an understanding of this virus in Asia and Africa[28]. The result of HHV-8 genome detection was highly relevant with surgical and dental operation, blood transfusion,

tattooing, cupping, drug used, number of blood donation and also with sexual relations suggesting various routes of viral transmission.

Six main genotypic clades (A to F) of the virus have been identified as having distinct distributions between different geographic and ethnic groups, and appear to transport with the populations[29]. Our results showed that the majority of blood donors had both Genotype A and C which coincided with most studies in neighbouring countries such as in Saudi Arabia[30], in Kuwait[31] and reported among Iranian patients[12,] and in other[29]. It's worth noting that both genotype A and C appeared about thirty-five thousand years ago in Europe and northern Asia[32] and it's widely spread around the world and including Middle East and Asia[29].

Genotype B was identified in our study and this type was mainly detected in Africa[28]. Genotype analysis may suggest transmission routes of the infection from other countries by travels[29]. Subtype D has been reported in Japan, china and Australia and only detected in non-AIDS patients[28] this may the reason that why we found it in the healthy blood donors. Genetic conditions can explain this finding and particular specific cell receptors could be responsible for the different susceptibility of some populations to this viral infection[33].

Mixed genotypes were identified in our study, and contained in various researches[34,35] rarely underlined among Genotyping studies[36], and may suggest cross-ethnic transmission of viral types[34]. The key question is whether genetic variety of KSHV patterns in living people reflects past demographic processes such as drift and host migration, or whether some KSHV genetic patterns have evolutionary advantages that raise their transmission or pathogenic ability[37]. This suggests that these genotypes were introduced into Iraq at multiple times and from numerous locations.

In the current study, genotypes were significantly related to age but not to sex, and the detection of genotypes has been heavily associated with the residence of blood donors; this coincided with Chinese study in north-western China[38]. Most of the areas recorded in our study are from popular areas and are known to be overcrowded with low income people, undereducated and lack of awareness, this will increase the possible risk of HHV-8 transmission especially during practices that expose them in the childhood period to the saliva of the infected persons such as sharing foods and dishes with pre-mastication of food, this was recorded in different studies as a route of transmission in endemic rural regions[39,40]. Also, the increase of HHV-8 prevalence was reported when both parents are positive and living in large families with siblings of close ages[41].

In conclusions, The present study provides further evidence that blood transfusion carries a potential risk for HHV-8 transmission, since that the percent of molecular detection were high among Iraqi blood donors, many risk factors play an important role in the HHV-8 transmission. Genotyping results revealed the domination of type A and C, in addition to significant association with age and residence of blood donors. Further studies are needed to explain plausible existence of HHV-8 infection among Iraqi population, which helps to explain many of unclear aspects of HHV-8 epidemiology, viral transmission factors and the related diseases.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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