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Detection of Epstein - Barr virus Type1 and Type2 in Subtypes of Non- Hodgkin Lymphoma in Kerbela Province by Using Polymerase Chain Reaction.

التحري عن فيروس الابسن بار النمط الاول النمط الثاني في الانواع الفرعية للاورام اللمفاوية اللاهودجكن في مدينة كربلاء بواسطة تفاعل الكوثرة .

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Abstract

Epstein-Barr virus (EBV) is a ubiquitous virus associated with lymphomas. However, in Kerbala as other parts of Iraq, EBV detection is not a part of the workup for lymphoma diagnosis.

. Archival formalin-fixed paraffin- embedded blocks from 44 patients with mean age 45.9±22.8 (range from 4 to 86years) diagnosed as Non Hodgkin lymphoma during the period from 2010 to 2018 in Kerbala city included in this study. Histological diagnosis was reviewed. Around 25mg of archival tissue were used to extract DNA. DNA extracts were used Polymerase Chain Reaction (PCR) targeting the EBNA3C region of the viral genome. This PCR system offered detection and typing of the EBV. T-test and chi-square were used to evaluate the association between age with the subtype of Non Hodgkin lymphoma, gender and EBV state.

The majority of the Non Hodgkin lymphomas(NHL) belonged diffuse large B cell (DLBC) [n=28, 63.6%], T-cell rich Diffuse large B-cell [n=9, 20.5%], high grade B cell Burkitt- like (BL) [n=3, 6.8%], small Blymphocytic [n=2,4.5%],high grade B cell(CD20-ve,CD30-ve[n=1,2.3%] and peripheral T-cell [n=1,2.3%]. The other subtypes of NHL were not reported in this study. Male and female were equal affected with NHL (22 for each) and the peak age range of NHL over 60 years. EBV was detected in 21(47%) out of all cases. EBV type 1 was more common to EBV2in all subtype of NHL. To our knowledge, this is the first- time such data has been generated in Iraq. In this study present evidence that Non- Hodgkin lymphoma cases in Kerbela are associated with EBV1 and EBV2 types. There is a difference in the distribution of the EBV types according to the subtypes of lymphoma. The aim of this study: to detect types of EBV in the subtype of Non- Hodgkin lymphoma (NHL) among patients from Kerbala province, Iraq

Keywords: Epstein Barr virus type1 and type 2, Non Hodgkin lymphoma subtype, molecular detection.

المستخلص

يعتبر فيروس الابسن بار فيروس شائع الانتشار ويكون عادة مرتبط بالأورام اللمفاوية. ومع ذلك ، في كربلاء وفي مدن العراق الاخرى ، لا يعتبر كشف الفايروس جزءًا من عملية تشخيص الإصابة بالورم اللمفاوي. تهدف هذه الدراسة التحري عن مدى انتشار أنماط الفايروس في الانواع الفرعية من الاورام اللمفاوية اللاهودجكين بين المرضى من محافظة كربلاء ، العراق. استخدمت الشرائح النسيجية المثبتة بالفومالين والمغمورة بالشمع لـ 44 مريض بمتوسط العمر 45.9± 22.8 (تتراوح اعمارهم من 4 إلى 86سنة) المشخصين بورم الغدد اللمفاوية الاهودجكين خلال الفترة من 2010 إلى 2018 في مدينة كربلاء تم دراستها. تم مراجعة التشخيص النسيجي من قبل مختص الانسجة . استخدم حوالي 25 ملغم من المقاطع النسيجية المخزونه لاستخلاص الحمض النووي. تم استخدام مستخلصات الحمض النووي في تفاعلات الكوثرة باستهداف منطقة الجين *EBNA3C*في الجينوم الفيروسي. استخدم التفاعل لتشخيص الفايروس و تميز انواع فايروس الابسن بار .تم استخدام اختبار تي و مربع كاي لتقييم الارتباط بين العمر بالانواع الفرعية من سرطان الغدد اللمفاوية اللاهودجكين والجنس و مقارنتها مع نتائج تشخيص الفايروس ..اظهرت النتائج ان غالبية الأورام اللمفاوية اللاهودجكين بانها تنتمي إلى النوع الفرعي *DLBC* حيث كانت 28 (63.6%)حالة ثم النوع الفرعي المختلط صغير وكبير *T-cell rich Diffuse large B-cell* في 9(20.5%) حالة ، و البركت لمفوما *BL* 3(6.8%)و واحد حالة لكل من *HGBC* و *PTC*. و لم تسجل حالات من الأنواع الفرعية الأخرى من الاورام الهوجكن في هذه الدراسة. الذكور ، الإناث نفس عدد الاصابات بالاورام اللمفاوية اللاهوجكن (22) . كانت الفئة العمرية اكثر من 60 عاما الاكثر اصابة بالاورام اللمفاوية اللاهوجكن . تم الكشف عن الفايروس في 21 (47%) حالة من جميع الحالات. كان *EBV1* أكثر شيوعًا في جميع الانواع الفرعية من النوع *EBV2*. على حد علمنا هذه هي المرة الأولى التي يتم فيها تسجيل مثل هذه البيانات في العراق. كانت اهم الاستنتاجات ان كثير من حالات الاصابات بالاورام اللمفاوية اللاهوجكن مرتبطة مع فايرس الابسن بار .هناك اختلاف في توزيع أنواع الفيروس وفقا لأنواع سرطان الغدد اللمفاوية.

INTRODUCTION

Epstein–Barr virus (EBV) belongs to the group of gamma-herpes viruses and was the first recognized human oncovirus [1]. It infects at least 90% of the population worldwide [2]. EBV associated with several diseases whose incidence varies dramatically in different parts of the world [3]. EBV is associated with different malignancies in different geographic regions remains puzzling and may be related to EBV genotypic variability through specific disease and geographic associations[4]. For instance: gastric and nasopharyngeal carcinoma in the east Asia (china, democratic people's republic of Korea and Taiwan) [5], Burkitt's lymphoma is a common cancer of children in equatorial Africa and Papua new guinea [6] and infectious mononucleosis (IM) adolescents/young adults from western societies [7]. EBV-associated lymphomas include Non-Hodgkin lymphoma(NHL) and classic Hodgkin Lymphoma (cHL), lymphomas arising in aids patients, immunocompromised individuals, peripheral T-cell lymphomas, angioimmunoblastic T-cell lymphoma, extranodal nasal-type natural killer/T-cell lymphoma, and other rare histotypes [8]. There are two type of EBV, EBV type 1 (EBV1) and EBV type 2 (EBV2). EBV2 varies genotypically from EBV1 in key latency genes [eg, those encoding EBV nuclear antigen 2 (EBNA2), EBNA3a, and EBNA3c][9].

The NHLs are a heterogeneous group of lympho-proliferative malignancies, consists of many subtypes, each with distinct epidemiology, etiology, morphologic, immunophenotypic, and clinical features **[10].** NHL is the 8th most commonly diagnosed cancer in men and the 11th in women in the world **[11]**. The NHL make up around 90% of all malignant Lymphomas **[12]**. NHL consists of over 60 subtypes, ranging from slow-growing to very aggressive. The three largest subtypes are Diffuse Large B-cell Lymphoma (DLBCL), Follicular lymphoma (FL), and Chronic Lymphocytic Lymphoma / Small Lymphocytic lymphoma (CLL/SLL) **[13].** DLBCL is the most common subtype of NHL, constituting 25–30% of adult NHL , FL accounts for approximately 15–20% of adult NHL,BL constitutes 1–5% of adult NHL in Western countries **[14].** The NHL constitutes the in the developed world it ranks seven. The most common NHL subtypes by far in developed countries (disregarding CLL and plasma cell entities) are DLBC lymphoma about 30% and FL about 20% **[12]**. In Africa countries DLBC is the commonest lymphoma (38.2-55%), BL shows that the endemic type is mainly confined to equatorial Africa and has a very close association with the EBV, while the sporadic variant shows only a 20% association with EBV and is seen mainly in

Europe and North America **[15]**. In Iraq ,age specific incidence rate for NHL by gender 2.88% for male and 1.77% for female in all top cancer in 2009 **[16]** than in 2011 the incidence rate for NHL, 6.8% for male and 4.48% for female in all ten top cancer **[17].** In Kerbela city, NHL record 6.09% as the fifth from ten commonest cancers in 2009 **[16].** Therefore aim of this study: to detect EBV type1 and type 2 in subtype of NHL among patients from Kerbela province by using polymerase chain reaction.

MATERILS AND METHODS

This was a laboratory-based retrospective cross-sectional study on archival formalin-fixed paraffin- embedded lymphoma tissue stored from April 2010 to March 2018 in Kerbala city. The study was accomplished at the medical research laboratory of medicine collage of Kerbala University. Al-Kafeel super speciality Hospital, Al-Hussein Teaching Hospital and Al-Sajad laboratory are the sites of helping for the collection of samples. All cases diagnosed as lymphoma depending on clinical history, histopathological examination and immunohistochemical finding.

From forty-four patients with Non Hodgkin lymphomas, archival FFPE tissue stored from April 2010 to March 2018 were collected and studied. The histopathology report of these cases included: all age groups which ranged from 4 years to 86 years old at diagnosis, gender (male22 and female 22), subtype of NHL and different anatomical tissue were obtained (cervical 27 , groin 5 axillary 4,abdominal 2, tonsil2,and 1 for submandibular, kidney, pharyngeal and intestine).

Sections of tissue block were obtained by the cut at 35- micron thickness by the microtome in histopathology laboratory in Al-Hussein Teaching Hospital. Those tissue sections were transferred to labeled sterile containers. Sterile blades and workplace were kept each sample. After each section, the microtome and workplace were disinfectants with cotton and bleach 10% in distilled water **[18]** to avoid the contamination of samples. Gloves were used in all steps of working.

Up to 25 mg tissue sections of FFPE put in 1.5 ml microcentrifuge tube. DNA was extracted by using gSYNC™ DNA Extraction kit (Cat.No.GS100 Geneaid Biotech Ltd. Korea) Purified DNA extraction stored at deep freeze -20 ℃.

For the purpose of ensuring the purity of the DNA and the absence of any inhibitor for the process of amplification by using PCR, the human □-actin gene (housekeeping gene) was used as internal control (Table 1). For accomplished amplify the gene fragment, add 1µl DNA primer (conc.10 Picomole) for each (Forward primer: 5‘-GCCATGTACGTTGCTATCC-3‘ and Reverse primer: 5‘-CCGCGCTCGGTGAGGATC-3‘) primers were obtained From(Bioneer, Korea), 2µl genomic DNA as template and 16 µl free nuclease water with 5 µl maxime PCR PreMix. The final volume was 25µl. The PCR mixture was done by Thermal Cycler started with initial denaturation at 95 ℃ for 2 minute, followed by 35cycles including: denaturation at 94 ℃ for 30 second, annealing at 55 ℃ for 1 minute and extension at 72 ℃ for 1 minute flowed elongation at 72 ℃ for 7 min yield than hold 4℃ for indefinite time. Final product was 200 bp. From amplified product, 5µl carry in agarose gel [1.5 gm agraose per 100 ml Tris/ Borate/ Ethylene Diamine Tetra Acetic acid (TBE buffer)] with 1µl ethidium bromide (10 mg/ml) on TBE buffer] by Horizontal Electrophoresis at 70 volts for 60 min. A100 bp ladder suitable for use as molecular weight standards for electrophoresis. Gel visualized by micro DOC Gel Documentation system and documenting the results in photography picture.

Used specific primer to detection EBV and determine the types by PCR technique. Specific prime for EBNA3C gene was used for this purpose. The sequence of this primer is 5‘-AGAAGGGGAGCGTGTGTTGT-3‘for Forward and 5‘-GGCTCGTTTTTGACGTCGGC-3‘for Reverse. Final product yield 153bp for EBV type 1 and 246bp for EBV type 2. The PCR amplification accomplished by 1.5 µl each primer (conc.10pM), 6µl genomic DNA as a template and 11µl nuclease-free water with 5µl maxime PCR premix kit. Final volume 25µl. PCR condition started with initially denaturation at 95℃ for 2 minutes followed by 35cycle include denaturation at 94 ℃ for 30 seconds, annealing at 55 ℃ for 45 seconds and extension at 72 ℃ for 1 minute. Finally, elongation at 72℃ for 7 minutes produces than hold 4℃ for an indefinite time. From final amplified product carried 5µl in agarose gel [1.5 gm agrose per 100 ml TBE buffer with 1µl ethidium bromide

(10 mg /ml) on TBE buffer] by Horizontal Electrophoresis at 70 volts for 60 minutes. A100 bp ladder was suitable for use as molecular weight standards for electrophoresis. Gel visualized by micro DOC Gel Documentation system and documenting the results in photography picture.

Data were analyzed with Statistical Package for the Social Science (SPSS) Software Version 22 for Windows. T test and chi-square were used to determine statistically association between EBV state with age, age group and subtype of NHL.

This study is restricted by working at the laboratory and there is not close contact with patients. Ethic's approval was obtained from Kerbala Health Directorate number 3522 on 11 December 2017. All wasted materials and pieces of equipment were collected, packaged and delivered to the Al-Hussein Teaching Hospital for a good and safe disposable by burning.

RESULTS

All the NHL blocks which were 44 samples with mean age 45.9±22.8 stored from April 2010 to March 2018 that documented by histopathology report as NHL included in this study (Table 2). NHL was affected 22 out of 44(50%) male with mean age 36.3± 24.6 and 22(50%) out of 44 with mean age 55.4±16.5 female (Figure1). NHL most affected age group were those aged group over 60 years (Figure2). Of all cases, DLBC was reported 28(64%) with mean age 55.7±17.8, T-cell rich Diffuse large B-cell 9(21%) with mean age 34.0±19.7, BL 3(7%) with mean age 5.6±1.5 and 4(8%) for other subtype (Table 3).

Beta-actin (a housekeeping gene) was used as internal PCR control to ensure the efficiency of the DNA extraction procedure. Specific primers were used to amplify 200bp fragments. Figure 3 showed 200 bp amplified products were successfully amplified from all DNA extracts. Both type EBV1 and EBV2 were detectable by amplified fragment 153bp for EBV1and 246bp for EBV2 by targeted EBNA3C gene (Figure 4). Of all 44 NHL analyzed in this study, DNA of EBV was detected in 21 (47.7%) with mean age 45.4±22.9 while 23(52.3%) with mean age 46.5±23.3were EBV negative (Figure5) and (Table3). Of all EBV detection in NHL, 12(57.1%) were DLBC, T-cell rich Diffuse large B-cell 5(23.8%), BL2 (9.5%) and 1(4.8%) for each Small B-lymphocytic (SBL) and high grade B cell (CD20 -ve , CD3 -ve) . In NHL positive result for EBV, EBV1 was detected in 13 cases and 8 for EBV2 (Figure 7).

Table 1: Sequence of primer that used in this study and there NCBI reference

Gene	Primer sequence		Product size (bp)	NCBI Reference
□-actin	F	GCCATGTACGTTGCTATCC	200	AK223055.1
	R	CCGCGCTCGGTGAGGATC		
EBNA3C	F	AGAAGGGGAGCGTGTGTTGT	153 For EBV1 246 for EBV2	NC_007605.1 NC_009334.1
	R	GGCTCGTTTTTGACGTCGGC		

Table2: characteristic of all NHL cases.

Type of lymphoma	N	Mean age ±SD	Gender	N	Mean age ±SD
NHL	44	45.89 ±22.868	**Male**	22	36.32 ±24.610
			female	22	55.45 ±16.532

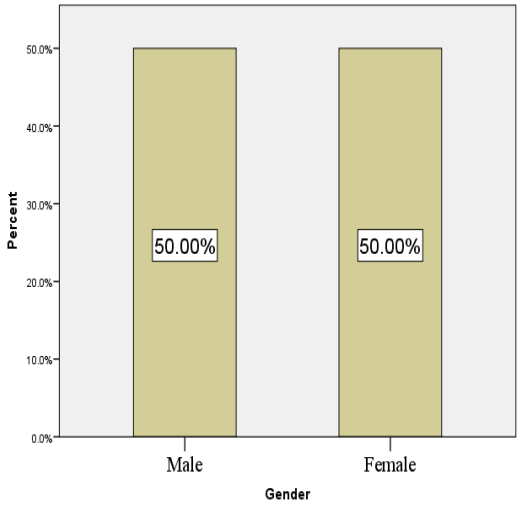


Figure1: distribution of gender in NHL in this study

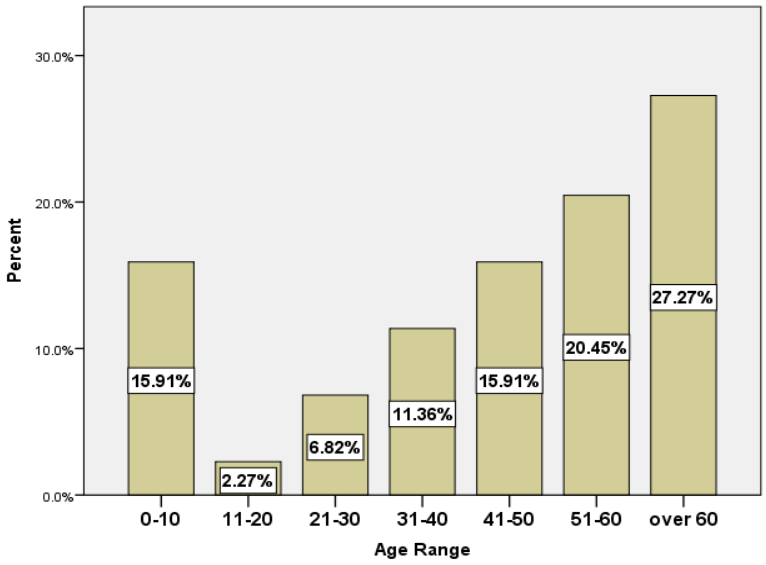


Figure2: Distribution of age range in NHL

Table2: characteristic of subtype of NHL in this study

Subtype	N (percent)	Mean Age ± SD
diffuse large B-cell (DLBC)	28 (64%)	55.8±17.8
T-cell rich Diffuse large B-cell	9 (21%)	34.0±19.7
High grade B-cell, Burkitt-like(BL)	3 (7%)	5.6±1.5
Small B-lymphocytic (SBL)	2 (4%)	45.0±21.2
high grade B cell (CD20 -ve , CD3 -ve)	1 (2%)	9.0
Peripheral T-cell	1 (2%)	35.0
Total	**44 (100%)**	**45.9±22.8**

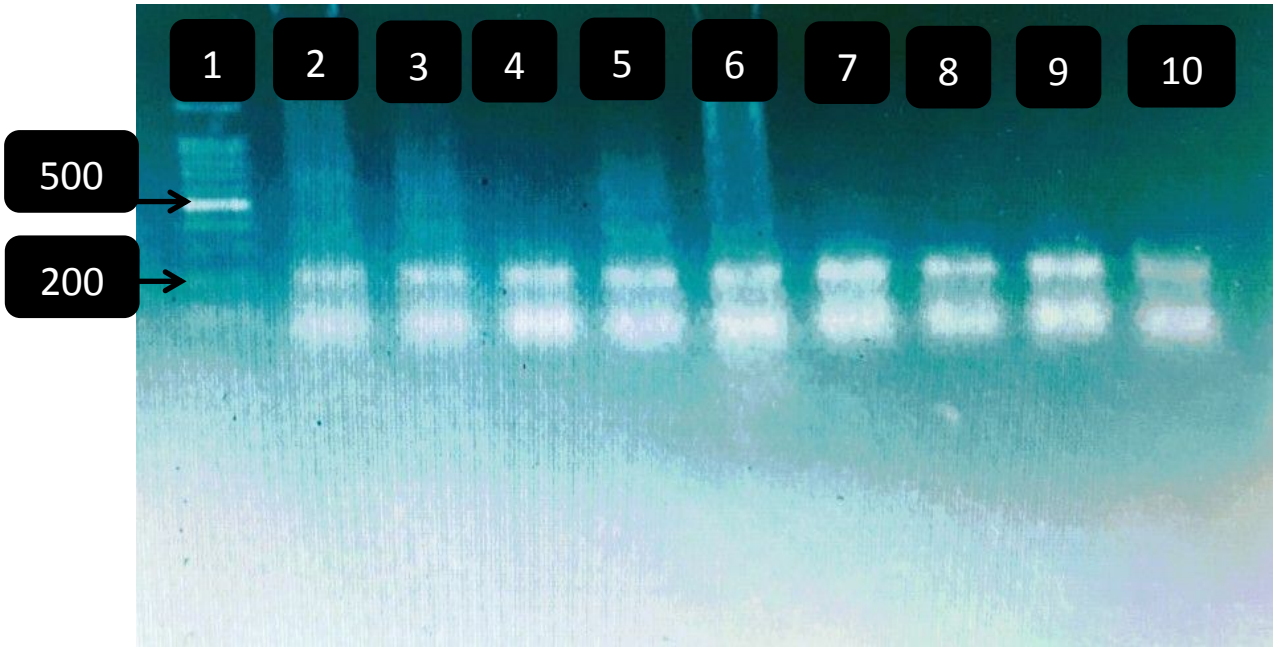


Figure 3: □-actin (control) in selected sample. Lane 1, 100bp DNA ladder, lane2, 3, 4, 5, 6 positive results for EBV1. Lane 7, 8,9,10 positive results for EBV2

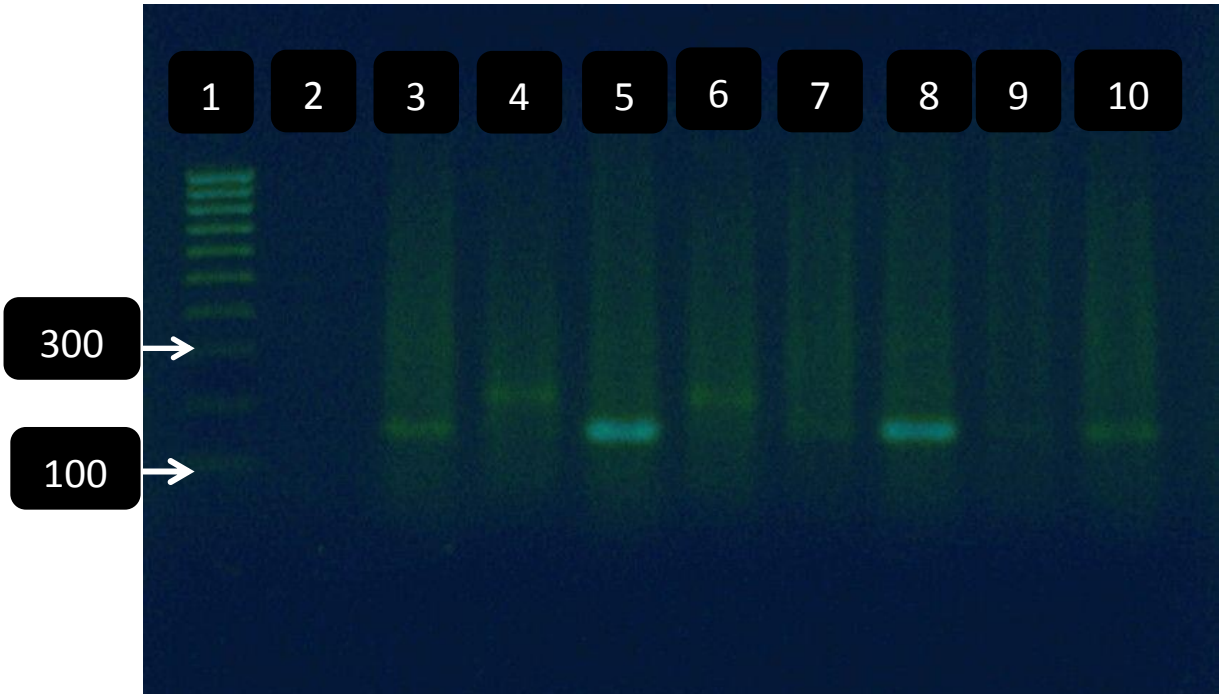
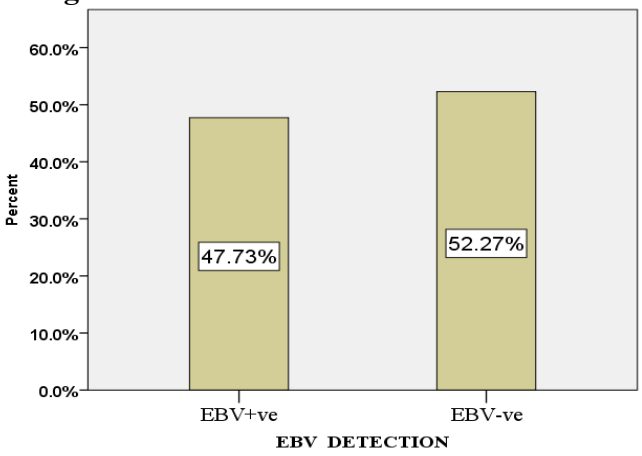


Figure 3.8: EBV detection and subtyping. Lane 1, 100bp DNA ladder .Lane2 negative control , lane 3positive control for EBV1,Lane 4positive control for EBV2,Lane 5,7,8,9,10 positive result for EBV1. Lane 6 positive e results for EBV2.

Figure 5: EBV DNA detection in all NHL



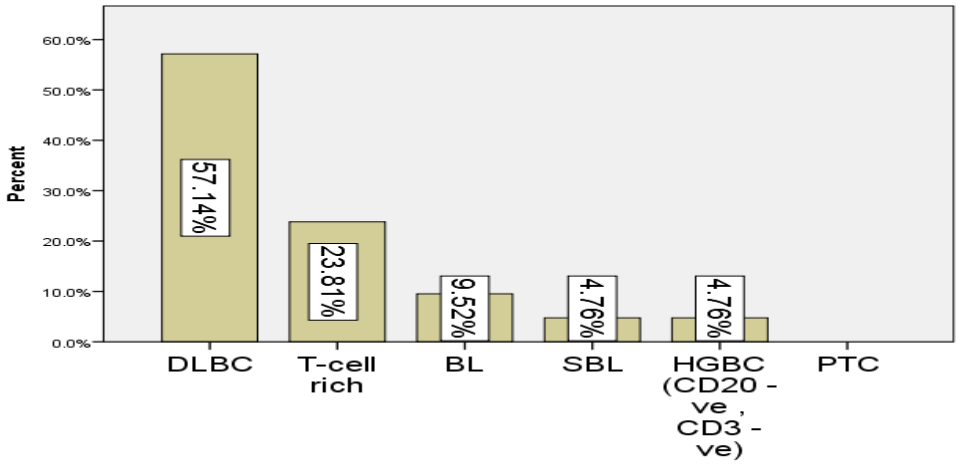


Figure 6: EBV DNA detection in all NHL subtype

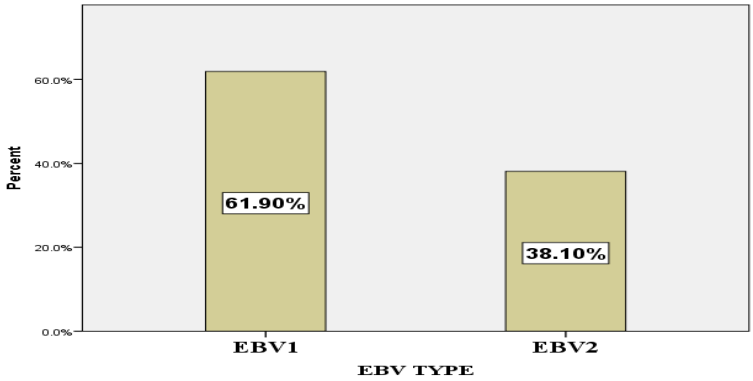


Figure7: distribution of EBV type1 type 2 in NHL

Table 3A: Distribution of mean age of EBV in NHL

NHL	EBV Result	№.of cases	Mean Age	Std. Deviation	T.Test Result Sig.
	EBV +ve	21	45.24	22.897	0.860*
	EBV -ve	23	46.48	23.339	

*Non-statistically significant (p= > 0.05)

Table 3B: comparison of age according EBV types

	EBV TEST	EBV CASES	№.of cases	MEAN Age	Std. Deviation± ±	T.TEST RESULT sig.
NHL	EBV1 vs. EBV2	EBV 1	13	48.62	±23.013	0.403*
		EBV 2	8	39.75	±23.119	
	EBV1 vs. EBV -ve	EBV 1	13	48.62	±23.013	0.792*
		EBV -ve	23	46.48	±23.339	
	EBV2 vs. EBV -ve	EBV2	8	39.75	±23.119	0.487*
		EBV -ve	23	46.48	±23.339	

*Non-statistically significant (p=> 0.05)

DISCUSSION

Epidemiologic, serologic and molecular studies have all suggested that EBV is involved in the development of a significant proportion of lymphoma [19]. The identification of strain variants is an effective approach to investigating virus transmission and persistence [20]. Diagnostic information regarding the prognosis and treatment of EBV-associated malignancies was more dependent upon the accurate morphological classification of these tumors than on whether EBV was identified [21].

The peak of the incident of NHL in this study was at the age range of over 60. This was consistent with studies and statistics in Iraq **[16]**, Iran **[22]**, Saudi Arabia **[23]** and in Central and South America **[24]**but was not consistent with some studies in Turkey **[25]** and Jordan **[26].** Males are at higher risk of developing NHL and the incidence rises steeply with age **[11].** There are 40 different subtypes of NHL which can occur at all ages; however the average age of diagnosis is in over 60 year and the incidence of this disease generally increased with age **[27].** The cause of NHL remains unclear, but it is related to immune deficiency, ionization radiation, and viral infection**[28].**

The DLBC was approximately two-thirds of the specimens which diagnosed as NHL followed T-cell rich Diffuse large B-cell then BL without any statistically significant association between NHL

subtype to age range and NHL subtype to gender (chi-square analyses failed because 95.2%, 83.3% of data have expected count less than 5 respectively). This result of DLBC consist with Iraqi study **[29]** Iranian study **[30],** Turkey **[31],** Algeria **[32],** Guatemala **[33]** central and south America **[34]** Far East **[35]** and Australia **[36] .** The DLBCL is the most frequent type of B-cell lymphoma, accounting for 30%-40% of NHL. The biological properties, genetic mutations, immune phenotypes, and cell morphology of DLBCL are varied, and therefore, DLBCL is considered a heterogeneous group **[37].**

. From the reviewing, the percentage of cases that EBV infection with NHL, the study find agrees with Iraqi studies which have reported of the EBV in NHL6(40%) out of 15 cases **[38]** and 14(43%) of all 32 cases **[39].** The results in this study are not compatible with several studies of Iraq's neighbors, for instant: Iran 9(26.5%) out of 34 cases **[40]** , Jordan 8(32%) out of 25 cases **[41]** and Turkey study 14(27.4%) out of 51 cases **[42].** Compare this study with Uganda study, here was a vast difference in the result , 87(79.8%) out of 109 cases **[43].** This large difference in results may be due to widespread of the EBV in Africa to earlier age of EBV infection and lower socioeconomic status have been related to increased EBV-associated BL in developing countries **[44]**. The NHL and EBV relation is weaker than the HL relation. However, the EBV plays an active role in the pathogenesis even in some types of NHL. The positive rates of EBV, indicate that EBV has a disease-predisposing role in a significant proportion of lymphoma patients; but it is noteworthy that socioeconomic conditions, genetic characteristics and immunosuppressive factors are important in the development of the disease as a whole **[42].**

Taking into consideration the size of the sample, subtype of BL more affected with EBV compare with another subtypes of NHL in this study. Depended on marked variation across different geographical regions with respect to age-specific incidence and primary tumor site **[45],** There are two forms of BL: endemic and sporadic **[46],** endemic BL diagnosed mostly in children, more frequently in boys than in girls observed south of the Sahara desert and in Papua New Guinea **[47]** while sporadic form mostly in children and adolescents observed outside Equatorial Africa and Papua New Guinea **[48]** all cases in this study of BL were endemic (age 3-10 years) and this result conduct with Western Iraqi study **[49]** but contrast with another **[50]**. This finding compatible with Iranian study**[51]** and Northeast Brazil **[52]**

From the reflection and reviewing the results in this study found that the type of EBV1 more affected than EBV2 in patients with NHL in Kerbala city without any statistically significant association between EBV types to the age range (chi-square analyses failed because 100% of data have expected count less than 5), EBV types to gender (chi-square analyses failed because 100% of data have expected count less than 5) and EBV types to NHL subtype (chi-square analyses failed because 100% of data have expected count less than 5). To the best of our knowledge, no study from the kerbala city and Iraq investigated the link between the type of EBV and NHL subtype. The tow types of EBV distinguished by the differences in EBNA-2gene, since the divergence in EBNA-2 reveals only 54% homology between the two types [53]. Interestingly, it was found that the EBV types noticeably differ in their transformation abilities. For instance, the EBV type 1 transforms the B lymphocytes more willingly than type 2 *in vitro*, and when a recombinant type 2 virus acquired the Type 1 EBNA-2A gene, it gained the transforming ability of type 1 virus [54]. One study suggest that EBV2 is an important human pathogen that present in a wider geographic distribution of HIV associated NHL than originally thought [55].

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