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Molecular Detection of Epstein - Barr virus and Human Herpesvirus-6 in a Sample of Iraqi Patients with Acute Leukemia

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ABSTRACT

The pathogenic roles of human herpesvirus-6 (HHV-6) and Epstein-Barr virus (EBV) in acute leukemia have been of great interest. Patients with leukemia should be evaluated for viral infection, so they could be diagnosed for optimal therapy. In the current study, we aimed to determine the frequency of HHV-6 and EBV in a sample of Iraqi patients with acute leukemia in children and adults before chemotherapy. Fluorescent probe-based quantitative polymerase chain reaction (Q-PCR) method was used to quantify copies of HHV-6 and EBV DNA in (ALL), 20 cases with acute myeloid lymphoblastic leukemia (AML), and 40 cases of hematological stable control subjects. Also, the effects of viral infection on hematological parameters were investigated. Results show that (47.5%) 19 out of 40 of patients at diagnosis recorded positive to one of the investigated viruses. Thirteen (32.5%) and 12 (30%) out of 40 patients with acute leukemia had positive EBV and HHV-6 viremia, respectively, while none of control group shows positive result with highly significant differences between patients and control groups (P<0.001). The mean EBV and HHV-6 viral load was (7737.615±9106.838 copies/ml) and (94393.58±214528.9 copies/ml), respectively. In this study, there was no significant association between viral infection and hematological parameters (P>0.05). In conclusion, infections or co-infections with EBV and HHV-6 could be a factor in the development of acute leukemia but further studies are required to establish whether there is a real association.

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INTRODUCTION

Leukemia is a rapid progression of cancer in bone marrow and other blood forming tissues leading to abnormal blood cells to the blood stream. Different types of leukemia occur in adults and in children. The real causes of leukemia are not understood until now that leukemia is a multifactorial disease, some genetic and environmental factors are suspected to participate in developing of the leukemia (Guan *et al.*, 2017).

Viruses such as, retrovirus and herpes virus families are etiological agents of human leukemia and lymphoma. Various epidemiological studies suggest

that increased leukemia rate associated with developing of abnormal immune response against infectious agents especially during the childhood (Kinlen, 1995). Human herpesvirus-6 (HHV-6) is a member of herpesviridae family, with double strand DNA (160-162 kbp in size) and linear genome.

HHV-6 was first isolated from patients with lymph proliferative disorders (Bolle *et al.*, 2005; Diamantopoulos *et al.*, 2018). HHV-6 integrates its DNA molecule in the cellular genes, this integration was reported in patients with acute lymphoblastic leukemia and noticed that this type of leukemia was transmitted from parents to their children which is found in about 1% of the general population (Daibata *et al.*, 1998; Faten *et al.*, 2012). The presence of HHV-6 and the expression of the viral DR7B on coprotein have been reported in Reed-Sternberg cells from patients with Hodgkin's lymphoma patients.

DR7 reported to bind with tumor suppressor protein p53 and lead to inhibition of p53 activated transcription (Faten *et al.*, 2012). HHV-6 latency is established in certain tissues, such as the salivary glands, T-cells and hematopoietic stem cells, and the virus can be reactivated in immune compromised hosts (Bolle *et al.*, 2005; Diamantopoulos *et al.*, 2018). Epstein-Barr virus (EBV) is one of the most common viruses that belong to gammaherpes virus; EBV has a linear genome with double-stranded DNA. The importance of EBV infection is the ability of the virus to become a latent virus after entering the body at any stage of life.

EBV infects 75% of the population before age 5, and most people are infected with adulthood (Kieff and Rickinson, 1996; Ahmed *et al.*, 2012). Regarding the viruses that have been proposed to play a role in the pathogenesis of acute leukemia, transforming viruses will integrate into the genome of precursor B cells, causing some disturbing in differentiation and proliferation stages of the cell.

The co-infection with EBV has suggested a probable linked with chronic lymphocytic leukemia in adults (Laytragoon-Lewin *et al.*, 1995; Sehgal *et al.*, 2010). In addition to that many common pathogens may stimulate indirectly an unusual response especially in genetically and immunologically susceptible children, leading to autonomous precursor B-cell proliferation. When the viruses are integrated into the genome of precursor B cells this will directly promote leukemogenesis by acting on differentiation and proliferation (Kinlen, 1995). So far according to our knowledge, no study is available in Iraq regarding detection rate and viral load of EBV and HHV-6 in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) in children and adults. The aim of this study was to detect the presence of EBV and HHV-6 in Iraqi patients with acute leukemia by quantitative real-time PCR.

MATERIALS AND METHODS

Study population

This study was approved by the Institutional Board Review committee (IBR) at Al-Nahrain College of Medicine, Baghdad, Iraq, written informed consent was obtained from each patient before conducting this study. Four (4) ml blood samples were obtained from 40 patients with acute leukemia (20 were ALL and 20 were AML) from Baghdad Center for Hematology in Medical City of Baghdad, Al Imamein Al Kadhimein Medical City and Central Teaching Hospital of Pediatrics in Baghdad. Specimens were taken from 20 children and 20 adults with acute leukemia before exposure to any therapy. All patients with leukemia were screened for hematological parameters: hemoglobin (Hb), packed cell volume (PCV), white blood cells (W.B.Cs), Neutrophil, lymphocyte.

In addition, blood samples were obtained from 40apparently healthy individuals from blood donating center in Al Imamein Al Kadhimein Medical City served as controls. The mean age of the patientswere16.85$\pm$13.37and 36.30 $\pm$23.31 years for ALL and AML patients, respectively (range, 2-68 years) and male to female ratio was 0.8:1. In this prospective study, all blood samples were obtained in sterile EDTA blood tubes, then at 1600 rpm for 20 min plasma separated by centrifugation, aliquot into two Eppendorf tubes and stored at (-40)°C until used.

The diagnosis of leukemia in all patients was developed using clinical, morphological, laboratory investigations including peripheral blast cells of more than 30%, bone marrow biopsy and fine needle aspiration cytology, and immunophenotypic criteria. Patients with secondary leukemia, hereditary hemolytic anemia and relapsing acute leukemia were excluded from this study.

DNA Extraction

Extraction of the viral DNA from plasma samples was done by DNA-sorb-B Extraction kit (Ref. K-1-1/B, Sacace Biotechnologies, Italy) according to instructions as follow: 10μl of internal control (IC) was added to 300 μl of lysis solution then100μl of plasma was added, mixed andincubated at 65°C for 5 min and then centrifuged at 12000g for 5 sec; 25μl of sorbent was added to each tube and vortexed for 5 sec, incubated at room temperature for 10 min and vortexed periodically. Then cen-

trifuged at 5000g for 1 min and the supernatant were discarded. 300μl of washing solution1was added vortexed vigorously, centrifuged at 5000g for 1 min and then the supernatant was discarded; 500 μl of washing solution2was added, vortexed vigorously, centrifuged at 10000g for 1 min, and then the supernatant was discarded. Washing step was repeated, and then the tubes were incubated with opened cap at 65°C for 5 min. After that, 50μl of elution buffer were added and incubated at 65°C for 5 minutes, vortexed periodically, centrifuged at 16000g for 2 min. Finally, the supernatant was collected, which contained purified genomic DNA, the purity of DNA extracts were measured using nanodrop (μLITEBioDrop, UK) and stored at -20°C until further analysis by QPCR.

Quantitative real time PCR

Fluorescent probe-based real time PCR method was used for qualitative and quantitative detection of HHV-6 and EBV usingHHV6 Real-TM Quant (Ref.V10-100FRT, Sacace Biotechnologies, Italy) and EBV Real-TM Quant.

Real time PCR was performed with the Strata gene Mx3005P by use of primers and probe sequences that amplified a fragment of each virus including a specific region. For HHV-6 DNA copies detection, polymerase gene of HHV6 was targeted while for EBV DNA copies detection, latent membrane protein-1 (LMP) was targeted. During the sample preparation, internal control (IC) was added to serve as an amplification control for each individually processed specimen and to classify potential reaction inhibitions. The viral-gene DNA amplification was detected on JOE (Yellow)/HEX/Cy3 channel, while exogenous internal control was detected onRox(Orange)/Texas Red Channel included both positive and negative control to check the reaction validity.

The device was set according to the manufacturer's instructions. The master mix was prepared according to the manufacturer's instructions. For one reaction final volume of 25μL was prepared as following: 10μL of PCR-mix-1, 5μL of PCR-mix-2 buffer and 0.5μL of HotStart DNA polymerase, and then, 10μL of DNA from samples/standards positive or negative controls were added to the mix.

The real-time thermal condition included holding step for 1 cycle as initial activation of Hot Start DNA Polymerase at 95oC for 15min. Then two cycling steps: the first cycling step includes 5 cycles of denaturation at 95C for 5 sec, annealing at 60oCfor 20 sec, and extension at 72°C for 15 sec, while the second cycling step includes40 cycles of denaturation at 95°C for 5 sec, annealing at 60oCfor 30 sec, and extension at 72°C for 15 sec with fluorescence data gathered during the second step (annealing step).

Interpretation of results

Real Time PCR instrument software interpreted the findings by crossing (or not crossing) the fluorescence curve with the threshold axis. The fluorescence is observed in JOE/Yellow/HEX/Cy3 and Red fluorescent channels ROX / Orange / Texas for EBV detection, although the fluorescence is observed in JOE/Yellow/HEX/Cy3 for HHV6 detection. For qualitative analysis, the samples were considered to be positive for DNA if the value of Ct is different from zero (Ct<35) in the channel JOE(Yellow)/HEX/Cy3, and they were interpreted as negative if the fluorescence signal is absent, the sensitivity of detection with EBV and HHV6 Real-TM Quantis approaching 200 and 100 copies/ml, respectively according to manufacturer. For each control and patient specimen, the concentration of EBV DNA copies/ml was calculated using the following formula,

$$EBV\,DNA\ copies/ml = \frac{EBV\,DNA\ copies/reaction}{ICDNA\ copies/reaction} \times IC\ coefficient$$

The concentration of HHV-6 DNA copies/ml was calculated using the following formula,

$$HHV6\ \ DNA\ \ copies/ml = HHV6\ DNA/reaction \times 100$$

Statistical analysis

Statistical package for social sciences (SPSS) version 23 was used for statistical analysis. Numerical data were presented as mean $\pm$ standard deviation, and comparison between means of study groups was done by using independent student t-test or ANOVA. Categorical data were presented as frequency and percentage, and chi-square test was used for comparison between frequencies of study groups. P value less than 0.05 was considered as significant.

RESULTS

The quantitative RT-PCR result shows EBV positivity in (32.5%) 13 out of 40 in patients with acute leukemia where (30%) 6 out of 20 in ALL group and (35%) 7 out of 20 in AML groups, while all control group shows negative result, as shown in Table 1. There was no significant differences between ALL and AML, however there were statistically significant differences between patients and control (p<0.05).

The result shows HHV-6 positive viremia in (30%) 12 out of 40 in patients with acute leukemia where (30%) 6 out of 20 in each of ALL and AML groups, while none of control group shows positive result

Table 1: Comparison of EBV statusin different study groups

EBV Infection		Patients with acute leukemia ALL Group	Patients with acute leukemia AML Group	Control Group
Negative	Count	14	13	40
	Row N %	70%	65%	100%
Positive	Count	6	7	0
	Row N %	30%	35%	0%
Total	Count	20	20	40
	Row N %	100%	100%	100%
P value between groups		0.0001		

Table 2: Comparison of HHV-6 status in different study groups

HHV-6 Infection		Patients with acute leukemia ALL Group	Patients with acute leukemia AML Group	Control Group
Negative	Count	14	14	40
	Row N %	70%	70%	100%
Positive	Count	6	6	0
	Row N %	30%	30%	0%
Total	Count	20	20	40
	Row N %	100%	100%	100%
P value between groups		0.001		

Table 3: Relationship betweenthe EBV, HHV-6 and the hematological parameters

Parameter (Mean ±SD)	EBV-Status Positive	EBV-Status Negative	P value	HHV-6 Status Positive	HHV-6 Status Negative	P value
	21.26 ±28.48	25.92 ±35.59	0.682	35.26 ±33.41	19.75 ±32.52	0.178
Neutrophils %	29.42 ±21.58	21.89 ±20.09	0.285	31.62 ±22.91	21.22 ±19.15	0.146
Lymphocyte %	61.82 ±22.13	51.94 ±27.13	0.261	57.15 ±24.84	54.29 ±26.54	0.752
Hemoglobin (g/dl)	7.83 ±2.16	8.06 ±2.24	0.758	8.08 ±2.25	7.94 ±2.21	0.859
PCV%	24.84 ±6.61	24.00 ±6.49	0.706	24.733 ±6.56	24.08 ±6.52	0.776

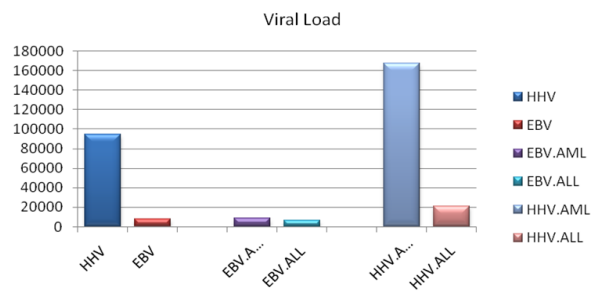

Figure 1: Comparison of mean HHV-6 and EBV viral load in different study groups

with statistically significant differences between patients and control (P<0.05), as shown in Table 2. In addition, (15%) 6 out of 40 of patients shows co-infection with both EBV and HHV-6. Interestingly, (47.5%) 19 out of 40 of patients positive to one of the investigated viruses either EBV &/or HHV-6 (data not shown).

Quantitative real time PCR (QRT-PCR) run gave positive EBV viremia in (32.5%) of patients, the mean copy number was (8895.714±12235.12; range: 422-10677) copies/ml in AML patients and (6386.5±3916.2; range: 600-35550) copies/ml in ALL patients. For HHV-6, the viremia was detected

in (30%) of patients, the mean copy number was (167701.2±296600; range: 420-770090) copies/ml in AML patients and (21086±19511.58; range: 266-40890) copies/ml in ALL patients. However, the mean EBV and HHV-6 viral load was (7737.615±9106.838 vs. 94393.58±214528.9) copies/ml, respectively, as shown in Figure 1.

Patients with acute leukemia were categorized into two groups according to the viral status. Then data were analyzed with student t-test, result shows that there was no significant difference according to hematological parameters between the EBV positive and EBV negative patients, neither between the HHV-6 positive and HHV-6 negative patients as shown in Table 3.

DISCUSSION

Various theories have indicated an infectious etiology of cancer, supporting either direct or indirect transformation mechanisms. Related pathways include viral oncogene expression along with cellular tumor suppressor gene deregulation, indirect mechanisms are mainly elicited by inflammation, mutagenic molecules production or immune suppression with lack of immune surveillance against cancer (Morales-Sánchez *et al.*, 2014).

Smith suggests that oncogenic viruses can infect immature lymphocytes during the first year of life and encourage leukemia through a process that is direct (Smith, 1997). In the present study we selected members of the herpesviridae family, EBV and HHV6 because they are lymph tropic viruses often transmitted in the first months of life. We found that (47.5%) 19 out of 40 of patients were positive by at least one of the viruses tested. Results revealed that 13 out of 40 (32.5%) of patients were positive for EBV-DNA, distributed as 6 out of 20 (30%) of ALL and 7 out of 20 (35%) of AML, on the other hand, all healthy control was negative Table 1.

This result is comparable to that reported by Ahmed *et al.* (2012) who found that the distribution of EBV LMP1 in pediatric leukemia patients is(42.6%),(33.3%)and (0%) in ALL, AML and control, respectively using conventional PCR technique (Ahmed et al., 2012). Several studies have noted that EBV may contribute to the pathogenesis of acute leukemia (Sehgal *et al.*, 2010; Ahmed *et al.*, 2012; Guan *et al.*, 2017). Other studies find no proof of EBV's involvement in ALL development (MacKenzie *et al.*, 2001; Morales-Sánchez *et al.*, 2014).

EpsteinBarr infects about 90 percent of adults and about 50 percent of children around the world (Guan *et al.*, 2017; Dunmire *et al.*, 2018). Exposure to oral secretions during kissing has been identified as the major source for primary EBV infection in adolescents and younger children (Dunmire *et al.*, 2018). However, primary infections by EBV have been reported in a small number of children with ALL in remission and the course of the infection did not appear to be influenced by either the underlying leukemia or chemotherapy (Look *et al.*, 1981). Since infectious mononucleosis (IM) has been documented as a preceding event in some cases of ALL as well as other lymphocytic neoplasms, it is possible that EBV may be able to activate or "switch-on" an oncogenic process in a manner similar to that described for murine leukemia (Levine *et al.*, 1972). In the present study, utilizing sensitive and specific technique like QPCR, this result found that (32.5%) of our patients had infected with EBV and the percentage might be higher than this due to the fact that we only detected LMP1 gene, which could detect the majority of cases but not all cases, that may bear a causal relationship. However, positive samples showed low viral load (8895.714±12235.12; range: 422-10677), Figure 1.

Such findings do not support an EBV's direct function in the development of Iraqi patients with acute leukemia. It is stated that lymphocyte chromosome mutations or translocation induced by an EBV infection can result in c-myc Oncogene activation and excessive expression, leading to lymphoma eventually (Grimm *et al.*, 2005). Although there is no theoretical link between EBV and the pathogenesis of AML (Sehgal *et al.*, 2010; Ahmed *et al.*, 2012; Guan *et al.*, 2017), however, EBV diagnostic tests is recommended in pediatric patients with AML (Creutzig *et al.*, 2012). The current study, HHV-6 was detected in 12 out of 40 (30%) of acute leukemia patients (viral load 94393.58 ± 214528.9 copies/ml), distributed as 6 out of 20 (30%) in each of ALL and AML patients, Table 2 and Figure 1. This is comparable to other studies (Hermouet *et al.*, 2003). Seror *et al.* (2008) reported that a total of 24.7% of ALL children were positive for HHV-6 genome. Hermouet *et al.* (2003) reported higher HHV-6 detection rate (36%) with a higher viral load in blood. In contrast, a study showed that HHV-6 was found in only 15% of patients with acute leukemia at diagnosis (Faten *et al.*, 2012). However, a study on pediatric ALL failed to reveal a causal relationship of the virus with ALL (Morales-Sánchez *et al.*, 2014). The differences in the frequency of HHV6 may be due to variability in patient cohorts, and to different qPCR sensitivities. Faten *et al.* (2012) found that HHV-6 was significantly more prevalent in blood and bone marrow at remission than at diagnosis which indicated viral reactivation after chemotherapy rather

than a causal role of HHV-6 in the genesis of acute leukemia Faten *et al.* (2012). However, the presence of HHV-6 is interpreted either as a secondary event of immune suppression, as an incidental finding, or as a causal for the disease (Diamantopoulos *et al.*, 2018). The current study showed that there was no significant difference according to hematological parameters and viral positivity at diagnosis of leukemia, Table 3, Guan *et al.* (2017) reported that among ALLs, WBC counts were higher in the EBV positive group than in the EBV negative group; this was caused by EBV infection that led to high WBC counts.

CONCLUSIONS

Our result could be explained by differences in samples size, viral load and stage of disease. There are many studies from Iraq on leukemia but to the best of our knowledge no previous study screened patients for EBV and HHV-6 before the induction of chemotherapy. In conclusion, this study detected EBV and HHV-6 nucleic acid in patients with leukemia, which is interesting finding in our community. Thus, in order to investigate the possible role of viral infection in acute leukemia, more molecular techniques with greater sample sizes are needed.

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