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Research Article

# Human Herpesvirus 4 or Epstein-Barr Virus Infections and Putative Role in the Cause of Childhood Leukemia

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## Abstract

**Background:** The exact cause of acute lymphoblastic leukemia (ALL), a severe cancer in Sudan, is still under assessment. Substantial progress has been made in understanding the contribution of infections to the aggressive transformation of B-cells, where Epstein-Barr virus (EBV) has indeed been reported as transformative triggers. The identity of an infectious agent remained anonymous. As there is no specific candidate agent in sight, we assumed that the EBV might be implicated in the underlying cause of leukemia.

**Methods:** A total of 90 participants were recruited for this study. Of these, 42 were ALL pediatric leukemia patients, 8 were positive-control patients with EBV-associated lymphoid malignancies and 40 were healthy individuals. They were tested for EBV-IgG antibodies. Only positive samples were detected by the PCR targeting the EBV BamH1W region.

**Results:** Tests have demonstrated the detectability of EBV DNA in a number of leukemia and control patients. Almost all subjects had EBV-IgG positive (> 85% of cases and controls) whereas EBV DNA was detected in 20% of cases (N = 42) and was not found in controls.

**Conclusion:** Our conclusion stated that there was no EBV DNA in all leukemic cells, and therefore most likely infection did not lead to ALL in Sudanese children.

***Keywords:*** *EBV; PCR; Leukemia; Diagnosis; BamH1W Region*

## Abbreviations

EBV: Epstein - Barr Virus; PCR: Polymerase Chain Reaction Assay; RICK: Radiation and Isotope Centre Khartoum; BL: Burkitt's Lymphoma; HD: Hodgkin's Disease; NPC: Nasopharyngeal Carcinoma; ALL: Acute Lymphoblastic Leukemia; CML: Chronic Myelogenous Leukemia

## Introduction

Epstein-Barr virus (EBV) is an etiological factor of infectious mononucleosis, Burkett's lymphoma, nasopharyngeal carcinoma (NPC) B- and T-cell lymphomas, Hodgkin lymphoma, and gastric cancer. The identity of infectious agents in acute lymphoblastic leukemia (ALL) has remained uncertain. Significant progress has been made in understanding the contribution of infections to aggressive B-cell lymphoma, in which Plasmodium falciparum, Epstein-Barr virus (EBV), Helicobacter pylori and Hepatitis C virus have indeed been identified as transformative triggers [1,2]. Growing evidence from epidemiological studies strongly indicates that the increased incidence of leukemia is likely to be due to (EBV) infections [3-5]. Viruses were proposed to play a role in the pathogenesis of ALL and there has been a high

incidence of Epstein Barr virus infection in acute lymphocytic leukemia in children [6]. Transforming viruses can be incorporated into the precursor B cell genome, disrupting differentiation and proliferation [4]. Alternatively, specific pathogens may function indirectly, inducing an unusual response in children who are genetically and immunologically susceptible, raising the proliferation of autonomous B-cell proliferation [3]. EBV infections were known to be the most likely cause of childhood B-cell precursor acute lymphoblastic leukemia [7]. EBV was the first human virus to be actually involved in carcinogenesis. It is a common infection affecting over 90% of the world's population [8]. The novel advances in molecular technology have permitted rapid and accurate quantification of the EBV genome. Real-time polymerase chain reaction (PCR) is a simple and accurate method for quantifying DNA and is commonly used not only as a diagnostic tool but also as an EBV-associated infectious diseases tool. Real-time polymerase chain reaction assay (q-PCR) has been commonly used in the plasma of leukemia patients for the identification of cell-free EBVDNA.

Recent studies have provided signs of EBV involvement in leukemia patients. Results suggest that an EBV DNA genome encoding the non-glycosylated membrane protein BNRF1 pl43 has been observed in a significant proportion of ALL patients. However, it was not possible to exclude the relationship between these viral infections and later childhood leukemogenesis in Sudan [9]. A large proportion of patients with acute lymphoblastic leukemia have demonstrated transcriptional activity of the gene EBV LMP1. EBV infections in lymphoid leukemia patients can be a factor that leads to the high incidence of pediatric leukemia in the Sudan [10]. There is no known single cause for all types of leukemia. Controversial theories have been proposed indicating the role of physical (i.e. high dose ionizing radiation, electromagnetic fields) [11,12], as well as chemical (i.e. benzene, formaldehyde) [13] and even biological factors (i.e. Down's syndrome) [14] responsible for incidents involving leukemia. Other approaches, such as detecting transcripts of EBV-RNA in tissues, can provide a broader understanding of transcriptional activity in latent and lytic EBV infections which may offer new insight into its pathogenic potential [15]. There is currently no US Food and Drug Administration (FDA) certified EBV DNA detection and quantification assay [16]. The Bam H1W is the most sensitive of the other primers for detecting low-level virus [17,18]. In addition, some studies have reported higher BamHI-W sensitivity than the LMP2 region. [19]. The causal relationship between EBV and leukemia has been extensively investigated in Sudan. However, there is no comprehensive analysis of any of the EBV subspecies referred to in this area. Interestingly, EBV plays a role in the etiology of several diseases such as cancer, rheumatoid and a co-factor for several autoimmune diseases. Logically, this trait leads to the belief that it is involved in leukemia-related incidents. In recent years, researchers have authenticated that EBV infection can cause leukemia but its mechanism remains unclear. Hence, we detected EBV BamH1W DNA sequences in patients with leukemia and healthy control subjects using a polymerase chain reaction (PCR) technique.

## Objectives of the Study

In this study, we have been looking for evidence of the association between EBV and leukemia in Sudanese children.

## Materials and Methods

### Enrollment of patients

The research has been conducted at the National Cancer Institute of the University of Gezira (NCIUG) and the Khartoum Radiation and Isotope Center (RICK) Sudan. The consent of the patients was requested by the Ethics authorities to obtain clinical material for research purposes. The Patients have previously been diagnosed with many diagnostic criteria, including cell morphology and flow cytometry.

### Sample collection

Plasma samples from 90 children with leukemia were obtained upon their consent.

### Sampling technique

The study was based on non-probability convenience sampling technique during admission to Radiation and Isotope Centre Khartoum (RICK) Sudan and the National Cancer Institute of the University of Gezira (NCIUG).

### Sample size

The sample size justification was computed according to the relationship pertaining to the study design:

$$N = \frac{(Z\alpha_{/2} \times \sigma)^2}{(ME)^2}$$

Where N = Sample size

$Z_{\alpha/2}$ = Confidence Interval (CI) = 1.96 for Confidence Level (95%).

$\sigma$ = The Standard Deviation from the mean = 1.99 in present study.

$ME$ = The chosen Marginal error to achieve in present study < 0.4.

The computed value for sample size = 95.08 rounded up to = ˜ 96 individuals.

### Extraction of DNA from the blood plasma

The DNA was extracted from the blood plasma using (G-spin TM Total DNA Extraction Kit) after the parents had given their consent. DNA extracted as follows: 200 µl of plasma into a1.5 ml micro centrifuge tube, 20 µl of proteinase K into sample tube and gently mix and add 200 µl of Buffer BL into upper sample tube and mix thoroughly, after that incubate the lysate at 56°C for 10 minutes. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the rid. Add 200 µl of absolute ethanol into lysate, and mix well by pipetting, carefully apply the mixture to the spin column, close the cap and centrifuge at 13,000 rpm for 1 minute. Discard the filtrate and place the spin column in a new 2 ml collection tube. Add 700 µl of buffer WA to the spin column without wetting the rim, and centrifuge for 1 minute at 13,000 rpm. Remove the flow-through and reuse the collection tube. Add 700 µl of WB Buffer to the spin column without wetting the rim, and centrifuge for 1 minute at 13,000 rpm. Discard the flow-through and place the column into a new 2 ml collection tube, then again centrifuge for additionally 1min to dry the membrane. Discard the flow-through and collection tube completely. Place the spin column into a new 1.5 ml tube and 50 µl of Buffer CE directly onto the membrane. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at 13,000 rpm to elute.

### Polymerase chain reaction

Polymerase chain reaction (PCR) was carried out for amplification using primers specific to a sequence within the EBV Bam H1W region. PCR assays were performed in a total volume of 25 µL for EBV 0.5 µL forward primer and 0.5 µl reverse primer (EBV-Bam*H1W* primer forward 5'GCA GCC GCC CAG TCT CT-3' Reverse5'ACA GAC AGT GCA CAG GAG CCT-3.). PCR was performed in a total volume of 25 µL for EBV 0.5 µL forward primer and 0.5 µl reverse primer, 1 µl of DMSO, Volume was completed to 25 µL per reaction mixed with 17 µL of D.W. The master mix of all samples was mixed by pipetting. Amplification was then set in an initial denaturation stage at 94°C for 10 minutes, then 40 cycles of 94°C for 45 seconds and annealing at 60°C for 1 minutes, followed by extension at 72°C for 45 seconds and a final extension stage at 72°C for 10 minutes ended the amplification (Techne TC-412 Thermal Cycler). PCR products (83 bp) have been

detected in 2% ethidium bromide-stained agarose gels in a horizontal electrophoresis apparatus. In each run, both a negative control contained DNA from a non-infected individuals and a positive control (a known size of EBV virus DNA) were tested.

### Statistical analysis

Data management was carried out using the Statistical Package for Social Sciences (SPSS®) version 22 for the assessment of the inter-relationship between exposure and disease of interest.

## Results

**Serology:** Antigen capture ELISA (Euroimmun, Germany) was used to assess EBV IgG antibodies to the viral capsid antigen VCA. VCA-IgG ≥ 22 AU/mL were considered to be positive (qualitative test). Tests were directed according to manufacturer's instructions. A total of 90 individuals have been recruited for this study. Of the total number of subjects under study EBV IgG antibodies were detected in 77 (85 per cent) using the ELISA kit. The EBV IgG detection rate was 34 out of 42 in leukemic patients, 35/40 in normal healthy controls, but zero in positive-control patients with EBV-associated lymphoid malignancies (Figure 1).

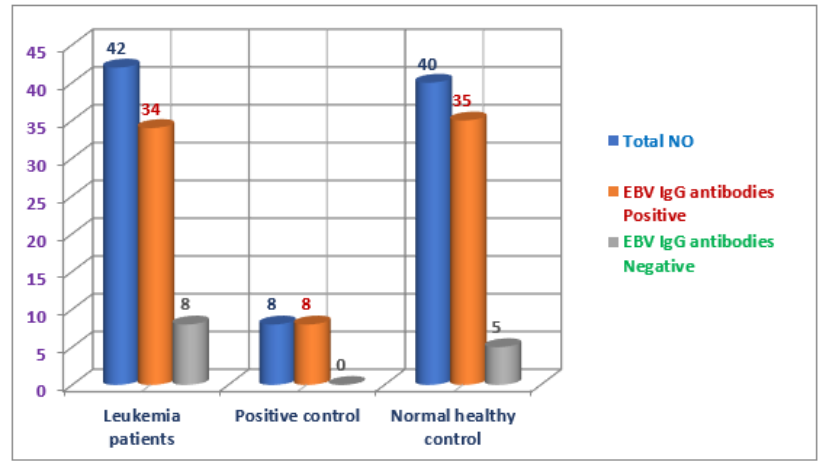


***Figure 1:*** *EBV serological response is seen with EBV Leukemia infection and controls.*

### Gel electrophoresis

The DNA was measured using 2% agarose gel electrophoresis. Five µl of the sample and 1µL of the DNA ladder were loaded into 2% agarose gel. The electrophoresis was carried out at 100 V 60°C for 40 minutes. The gel was then examined under UV light to visualize the DNA. The band size was determined by the migration of the DNA ladder and was captured in the gel documentation system. Figure 2 illustrate some of the PCR results. Forty two ALL pediatric patients that underwent chemotherapy treatment at (RICK) and (NCIUG). The plasma cell-free EBV DNA was successfully detected in (20%) during complete remission (CR (1), complete remission (CR (2), complete remission (CR (3). The correlation between EBV Bam H1W region and 42 (20%) ALL pediatric leukemia patients was significantly higher ($P < 0.01$; chi-square test) compared with the incidence in the control group (0 of 8 individuals; 0%). When we compared plasma EBV DNA levels in these patients, we found that the median titers were significantly higher ($P < 0.001$; Mann-Whitney rank sum test) in patients with leukemia.

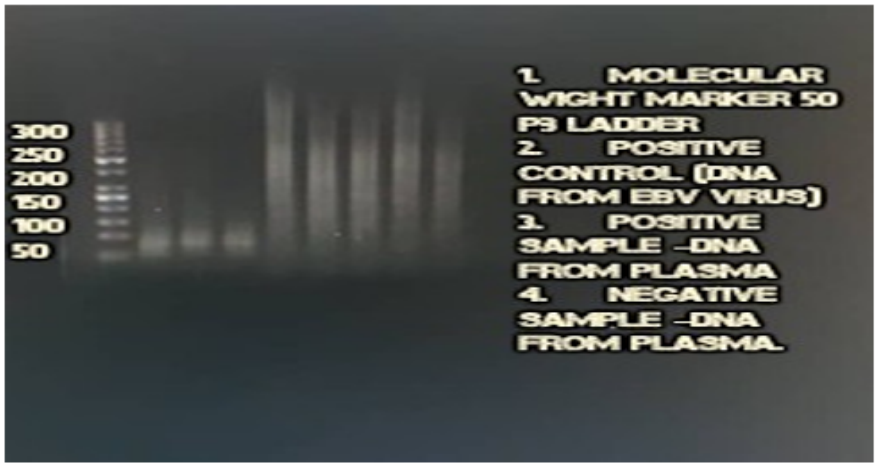


***Figure 2:*** *EBV DNA amplification from plasma sample of acute lymphoblastic leukemia (ALL) patients. Lane-1: Molecular Wight marker 50 pb ladder, Lane-2: Positive control (DNA from EBV virus), Lanes-3&4: Positive sample -DNA from plasma, Lanes-5-6: Negative sample -DNA from plasma. BamH1W gene Amplicon Size 83 bp.*

## Discussion

Acute lymphoblastic leukemia (ALL) is the most common form of pediatric cancer and has increased over the last few decades. EBV is a lymphotropic herpesvirus. It is highly prevalent and has been associated with multiple malignancies. The EBV-associated malignancies are an important cause of child mortality and morbidity, EBV is pathogenically associated with a well-defined lymphoid and epithelial tumors. The study have attempted to determine the presence of EBV infections in plasma samples from pediatric ALL patients. In recent years, some convincing evidence has been obtained concerning the causal relationship between EBV and a variety of childhood leukemias. Like other cancers, leukemia is the result of somatic DNA mutations. Mutations cause leukemia by activation of oncogenes or deactivation. These mutations may occur spontaneously or as a result of radiation exposure [11]. Among adults, ionizing radiation was the most common cause of leukemia. Other causes include ionizers, viruses and chemicals [12]. The study demonstrates the correlation between the plasma cell-free EBV and the leukemia in Sudanese patients. The findings were consistent with those of Haitham., *et al*, Ahmed., *et al*. and Sehgal., *et al*. [6,9,10] but not with Calvente I., *et al*, Corso A., *et al*, Austin H., *et al*. and Arico M., *et al*. [11-14]. Haitham., *et al*. observed that the EBV BNRF1 pl43 gene was present in a significant proportion in Sudanese pediatric leukemia patients. Where Calvente I., *et al*, Corso A., *et al,* Austin H., *et al*. and Arico M., *et al*. said studies so far have not convincingly confirmed or ruled out a correlation between EBV and the risk of childhood leukemia. In Finland, Matti., *et al*. identified a link between maternal reactivation of EBV and the development of ALL in children. We were motivated by the shortage of such relevant details conduct a systematic study of the circulating EBV DNA levels in multiples time points during treatment using a qualitative PCR assay. This research is backed by our observations and by the incidence of EBV in pediatric leukemia in Sudan. The rate of EBV in pediatric leukemia in Sudan may be higher than this since only the EBV-BamH1W gene was identified. Our samples were taken from those patients who received treatment, so the viral load of EBV may decrease to 0 Copies\cells upon completion of radiotherapy except in cases of cancer EBV is already known to be a causative agent such as NPC [20,21]. Kenny., *et al*. [22] also stated that all patients who responded to the therapy showed a significant reduction in plasma EBV DNA at low or undetectable levels. Disagreement may be due to the source of the cell-free EBV DNA and the type of samples selected for each study.

## Conclusion

Our conclusion stated that there was no EBV DNA in all leukemic cells, and therefore most likely infection did not lead to ALL in Sudanese children.

The study was approved by the Sudan Medical Specialization Ethics Review Board, Sudan.

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