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# Viral Infections Associated with Therapy in Pediatric Acute Lymphoblastic Leukemia

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## ABSTRACT

***Background:*** Acute lymphoblastic leukemia (ALL) patients are immunodepressed thus their immune systems are less able to fight off infections once acquired. Nosocomial infections can be transmitted by direct and indirect routes through equipments, supplies and hospital procedures.

***Aim of Work:*** To screen for post-therapy viral infection in pediatric ALL cases to verify whether treatment or hospitalization play a role.

***Patients and Methods:*** Forty four patients with ALL were enrolled in this study from August 2007 to September 2008, upon admission and at least 6 months after initiating chemotherapy from the Pediatric Oncology Department of National Cancer Institute (NCI), Cairo University. Their median age was 7.6 years with a male to female ratio of 1.2:1. Serum samples were collected and tested for viral markers for HCV, HBV, CMV, EBV and HHV-8 by ELISA and Real-Time PCR.

***Results:*** In Pediatric ALL cases, the rate of change of results from negative to positive for HBsAg, and CMV IgM, before and after treatment, showed a high statistically significant *p* value (<0.001), and a relative increase in HCV Ab and EBV IgM but did not reach a significant value. No change in positivity of results regarding either CMV IgG or EBV IgG. By Real-time PCR, the rate of change from negative to positive, in Pediatric ALL cases, showed a high statistically significant change before and after treatment in HBV, HCV and CMV (*p* value <0.001) and insignificant change for EBV and HHV 8 (*p* value >0.05).

***Conclusion:*** Pediatric ALL cases had a high significant prevalence of viral infections with HBV, HCV and CMV detected by real-time PCR when compared before and after treatment. This indicates the necessity of initial screening as well as re-screening after therapy.

***Key Words:*** *Acute lymphoblastic leukemia – Hepatitis C virus – Hepatitis B virus – Human herpes virus-8 – Cytomegalovirus – CMV – EBV – Real time PCR – Nosocomial infections.*

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) patients are immunodepressed thus their immune systems are less able to fight off infections once acquired **[1]**. Nosocomial infections can be transmitted by all modes of transmission, however, direct person to person transmission and indirect transmission through equipments, supplies and hospital procedures are the most common in hospitals **[1]**. The presence of multiple human herpes virus (HHVs), including Epstein Bar virus (EBV) and cytomegalovirus (CMV) at high loads is common in B-ALL and B-Cell chronic lymphocytic leukemia (B-CLL) samples **[2]**.

HHVs may disrupt immune functions and/or induce mutations in infected cells. Re-activation of presumably latent HHVs during the course of anti-leukemia treatment can occur, usually explained by suppression of immune functions in aggressively treated patients. Thus, an increased rate of positivity for HHV-6 A, HHV-8 and CMV in leukemia patients raises several questions, are these viruses found more frequently in leukemia patients because of a specific immune dysfunction toward HHVs? The processes of B-ALL or B-CLL induction may render affected individuals more susceptible to infection by HHVS or to their activation from latency **[3]**.

The aim of this work was to screen for post-treatment viral infection in immunocompromised children with acute leukemia to verify whether treatment or hospitalization play a role or it is a sort of reactivation of a dormant virus that was present before treatment. In addition,

we aimed to study the potential possibility of infection post-treatment after blood transfusion, or chemotherapy.

## PATIENTS AND METHODS

*Patients:*

From August 2007 to September 2008, forty four patients with ALL were enrolled in this study. All patients were recruited at the Pediatric Oncology Department, National Cancer Institute (NCI), Cairo University, Egypt, upon admission and establishment of diagnosis, and at follow-up at least 6 months after initiating chemotherapy.

Patients recruited were 24 males and 20 females with a ratio 1.2:1; the median age was 7.6 years (range: 7 months –17 years).

*Inclusion criteria:* New ALL cases whether associated with other pathological or neoplastic conditions or not, before chemotherapy and put under follow-up for at least 6 months. The pediatric age group was below 18 years old.

*Sample collection and storage:*

Two venous blood samples each 10ml were collected from each case at diagnosis and at least 6 months post-chemotherapy under aseptic condition, and each sample was divided into two tubes for serum preparation and blood on EDTA vacutainer. Sera were separated, divided into aliquots and stored at –80ºC; 2 ml EDTA blood were collected for CBC and flowcytometry, while 3 ml whole blood was separated into serum and cellular components by centrifugation for 15 minutes at 1200xg within 2 hours, in sterile polypropylene tubes. Stored serum samples at –80ºC were screened by ELISA and PCR to exclude previous old infection not related to therapy.

*Methods:*

All patients were tested for CBC, with differential leukocyte count and platelet count (EDTA blood samples performed on Sysmex K21) **[4]**. Liver function tests: Total bilirubin, ALT and AST measured using (ACE) clinical chemistry auto analyzer. Bone marrow aspiration for the diagnosis and follow-up of the patients with cytochemical stains to confirm the diagnosis was performed. Immunophenotyping by flowcytometry was done, using monoclonal antibodies, to establish the diagnosis.

*Tests for viral infections:*

Detection of Antibodies by ELISA for HCV-Ab, CMV IgM & IgG and EBV IgM & IgG was performed using Adaltis EIAgen (Italia S.P.A) kits in human serum and for HBsAg using Bioelisa HBsAg (Biokit, S.A. BARCELONA-SPAIN) as previously described **[5]**. Also, detection of HCV, HBV, CMV, EBV and HHV8, nucleic acid by Real-time PCR was performed using Real ArtusTM HBV, CMV, EBV and HHV8 kits (ABI PRISM® 7900HT Sequence detection system, Applied Biosystem).

*Nucleic Acid Extraction for HCV-RNA, HBV-DNA, CMV-DNA, EBV-DNA and HHV8-DNA:*

RNA extraction for HCV was performed using the QIA amp ® viral RNA Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany) **[6]**. One step RT-PCR for HCV RNA was performed by using Taq Man Gold RT-PCR kit (Applied Bio-Systems, USA); it contains Multiscribe Reverse Transcriptase for first strand cDNA synthesis and thermal stable Ampli Taq Gold DNA polymerase for second strand cDNA synthesis and DNA amplification. The RT-PCR thermal protocol was as follows: 48ºC for 30 min for Reverse Transcription step for RNA to cDNA, followed by 95ºC for 10 minutes for DNA polymerase activation, followed by 95ºC for 15 sec for denaturation step, ending by 60ºC for 1 min for PCR annealing and extension step for 50 cycles.

DNA extraction for HBV, CMV, EBV and HHV-8 was performed by QIAamp® viral DNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions **[6]**.

*Real-Time PCR for HBV-CMV-EBV and HHV-8:*

Nucleic Acid Detection by Real-Time PCR for HBV, CMV, EBV and HHV8 was done by Real Artus™ kits and sequence detection by ABI PRISM® 7900HT (Applied Biosystem, Foster City, CA). Thermal protocol for detection of HBV, CMV and EBV genomes were as follows: 95ºC for 10 minutes to allow Taq activation, followed by 50 PCR cycles 95ºC for 15 seconds for denaturation and 60ºC for 60 seconds for annealing and extension. The standard curve was prepared by serial dilution using the positive control starting from $2x10^{\wedge 7}$ copies per ul to 2 copies per ul.

*Statistical analysis:*

SPSS software package 12.0 for Windows (SPSS Inc., Chicago, IL) was used for data management. Kappa statistic estimates agreement beyond chance between variables with non-numerical values (qualitative outcomes). Kappa of value = zero indicates lack of concordance and when equal 1.0 indicates complete agreement. Chi-square test was used for testing proportion independence. McNemar test showed if there was any change in viral profile after treatment (ELISA, PCR). For virus results prior to treatment completely negative, virus positivity rate after treatment was tested against a hypothetical binomial distribution (0.999) for significance. *p* value is significant at ≤0.05, and highly significant at ≤0.001.

## RESULTS

*Clinical data:*

From August 2007 to September 2008, 44 pediatric patients with acute lymphocytic leukemia were recruited at diagnosis and after treatment for 6 months. Most of the cases presented with hepatosplenomegaly (78%), lymphadenopathy (88%), and bone pain with tenderness (80%). Blood Transfusion was reported in 97.7% of patients from 1 to 15 times (mean: 7 times) during follow-up (Table 1). All cases started chemotherapy shortly after establishment of diagnosis.

Table (1): Blood Transfusion frequencies in 44 ALL patients during follow-up.

| Frequency | No. of Cases | % of Cases |
|---|---|---|
| < 5 times | 14 | 31.81 |
| 5-10 times | 17 | 38.63 |
| > 10 times | 12 | 27.27 |
| 0 time | 1 | 2.3 |
| Total | 44 | 100 |

Liver enzymes were monitored before and after treatment. Before treatment, 43/44 (97.7%) of cases showed normal results and 1/44 (2.3%) of cases showed one fold elevation, while after treatment, 6/44 (13.6%), of cases showed one fold elevation, 12/44 (27.3%) of cases showed two folds elevation and 26/44 (59.1%) of cases remained with normal liver enzymes.

*Results of viral infections using serological markers before and after treatment:*

As shown in Table (2), HBsAg was detected in 29.5% of patients after treatment, while only 4.5% were positive before treatment, showing a highly significant change (*p* value <0.001). In addition, CMV IgM antibodies were positive in 4.5% of patients after treatment, while all were negative before treatment showing a high statistically significant difference (*p* value = 0.001).

The changes in the results of HCV Abs, CMV IgG, EBV IgM and IgG, before and after treatment were statistically insignificant.

*Results of viral infections by real time PCR before and after treatment:*

HCV RNA, HBV DNA, CMV DNA and HHV8 DNA by Real Time PCR testing were negative for all cases before treatment. EBV DNA was positive before treatment in one patient only (2.3%). After 6 months of treatment, the rate of change from negative to positive was detected for HCV RNA in 25%, for HBV DNA in 9% and for CMV DNA in 4.5% of patients; it was highly statistically significant (*p* value <0.001). On the other hand, detection of EBV DNA in 4.5% and HHV8 DNA in 2.3% of the 44 patients showed statistically insignificant change (*p* value >0.05) (Table 2).

Table (2): Results of Viral Serological Markers by ELISA and Viral Nucleic Acids By Real Time PCR before and after treatment.

| By ELISA | Positive ALL Cases (44) Before treatment Number (%) | Positive ALL Cases (44) After treatment Number (%) | *p* value |
|---|---|---|---|
| HCVAb | 1 (2.3%) | 3 (6.8%) | >0.05** |
| HBsAg | 2 (4.5%) | 13 (29.5%) | ≤0.001* |
| CMV IgM | 0 (0%) | 2 (4.5%) | ≤0.001* |
| CMV IgG | 42 (95.5%) | 42 (95.5%) | >0.05** |
| EBV IgM | 1 (2.3%) | 2 (4.5%) | >0.05** |
| EBV IgG | 44 (100%) | 44 (100%) | >0.05** |
| By Real Time PCR | | | |
| HCV RNA | 0 (0%) | 11 (25%) | ≤0.001* |
| HBV DNA | 0 (0%) | 4 (9.1%) | ≤0.001* |
| CMV DNA | 0 (0%) | 2 (4.5%) | ≤0.001* |
| EBV DNA | 1 (2.30%) | 2 (4.5%) | >0.05** |
| HHV8 DNA | 0 (0%) | 1 (2.3%) | >0.05** |

NB; *p* value = Significant ≤0.05 - highly significant ≤0.001
*: Significant. **: Insignificant.

*Concordance of Serological markers and Real Time-PCR results after treatment of ALL cases:*

By comparing results of RT-PCR and ELISA regarding HCV infection after treatment, it was found that 32/44 patients (72.7%) were negative and 2/44 patients (4.5%) were positive by both tests. However, 10/44 patients (22.7%) did not give the same results by both tests; 9 patients (20.5%) were positive by PCR but failed to be detected by ELISA while 1/44 patient (2.3%) was positive by ELISA, yet gave negative results by PCR (Kappa = 0.2 ($p$>0.05).

In HBV results after treatment, 30/44 (68.1%) of patients were negative, 3 cases (6.8%) were positive by both ELISA and Real time- PCR and 11 cases (25%) did not give the same results; one patient (2.3%) was positive by Real time PCR and failed to be detected by ELISA, 10 patients (22.7%) were positive by ELISA, yet gave negative results by RT PCR. (Kappa = 0.25) ($p$=0.037).

However, in both CMV and EBV testing, it was found that 42 patients were negative by both ELISA and RT PCR and two patients were positive by both ELISA and RT-PCR, and there was no difference in results by the two tests. Kappa = 1 ($p$<0.001).

Generally, there is significant concordance of Real time PCR and ELISA results regarding HCV ($p$=0.084 and Kappa = 0.2). High significant concordance in CMV and EBV results ($p$=0.00 and Kappa = 1) and regarding HBV ($p$=0.037: and Kappa = 0.248).

*Correlation between abnormal blood parameters and positive viral panels:*

Neither TLC, platelet count nor HB showed any relation to any of the 5 viral infections both by serological markers and Real time PCR ($p$ value >0.05).

*Correlation between elevation of liver enzymes and positivity of viral panels after treatment:*

Liver enzymes elevation and positive serological markers results showed significant association in cases of HCV Ab ($p$=0.01) and a highly significant association with HBsAg ($p$=0.005), and no association with CMV IgM ($p$=0.08) or EBV IgM ($p$=0.7).

However, there was highly significant association between positive RT-PCR results after treatment and elevated liver enzymes in HCV ($p$=0.006) and HHV8 ($p$=0.04) and no association in HBV, CMV, or EBV cases ($p$=0.4, 0.07 and 0.7 respectively).

*Correlation between frequency of blood transfusion and positivity by Real time PCR after treatment:*

In HCV RNA positive cases, results showed a highly statistically significant correlation with the frequency of blood transfusion ($p$=0.004). Although 4 cases were HBV DNA positive, no significant correlation was found between frequency of blood transfusion and positivity ($p$=1.0). Correlation between frequency of blood transfusion and positivity of CMV, EBV, and HHV8 could not be done due to small numbers of subgroups.

*Single and combined infection with different viruses by Real time-PCR and Serological markers:*

By ELISA, 25/44 cases (56%) were free of any viral infection, 17/44 (38.6%) showed single viral infection and 2/44 cases (4.5%) showed double viral infections. By Real time-PCR, 27/44 cases (61.4%) were negative, 17/44 (34.1%) showed single infection and 2/44 cases (4.5%) showed double infection.

By comparing combined positivity by serological markers and RT-PCR, 20 patients were negative by both, 10 had single infection combined by both ELISA and RT-PCR. On the other hand, 5 cases were negative by RT-PCR, but were found to have a single viral infection by serum markers and 2 cases were also negative by PCR and had double infections by serum markers. RT PCR showed double infections in 2 cases which showed single infection by serum markers. Fair agreement in co-infection results between ELISA and RT-PCR was encountered (Kappa = 0.385, ($p$=0.004).

## DISCUSSION

The current study was carried out on 44 patients newly diagnosed as acute lymphoblastic leukemia, recruited at the Pediatric Oncology Department - National Cancer Institute (NCI), Cairo University from August 2007 to September 2008 following admission.

Detection of viruses in the current study was done by both ELISA and real time-PCR (RT-

PCR) to assess viral infection after 6 months of treatment. Since a relatively low sensitivity was found if ELISA was used alone to detect viral infection, there was a need to establish additional Real Time-PCR (RT-PCR) testing for detection of viral RNA or DNA in order to identify all infected children. Tests by RT PCR are much more specific and are considered the gold standard for measuring current infection with viruses **[7]**.

Liver enzymes elevation concordance by ELISA results showed significant association with HCV Ab and HBsAg results, and insignificant association with CMV IgM and EBV IgM results. However, positive RT-PCR results after treatment and elevated liver enzymes showed highly significant association with HCV ($p<$ 0.001) and HHV8 ($p<0.05$) and insignificant association with HBV, CMV and EBV cases ($p>0.5$) results. Levels of hepatitis biochemical markers do not reliably reflect the severity of disease. Furthermore, a normal ALT or AST value does not indicate that the liver disease is inactive **[8]**.

The purpose of comparing serological markers with Real Time-PCR results was to detect the existence of new viral infections after 6 months of treatment, to relate this incidence to treatment (where RT-PCR closes the window period), and if there is a great possibility that any current infection detected was due to reactivation of previous old infection (detected by IgG for CMV and EBV) as a result of the immunosuppression effect of the disease and treatment.

Serological markers for HCV Ab were positive in 3 patients (6.8%) after 6 months of treatment, compared to one patient (2.3%) before treatment. However, by RT- PCR, HCV RNA was detected in 11 patients (25%) after 6 months of treatment, while they were all negative before treatment. By comparing results of RT-PCR and serological markers for HCV infection after treatment, it was found that 72.7% were negative and 4.5% were positive by both tests. However, 22.7% did not give the same results by both tests; 20.5% were positive by RT-PCR but failed to be detected by ELISA. On the other hand, only one patient (2.3%) was positive by ELISA, yet gave negative results by RT-PCR.

There is poor agreement of results between ELISA and RT-PCR. This could possibly be due to the window period where antibodies were not yet detected due to the immune-compromising effect of treatment. The use of Real time-PCR was able to detect the RNA of the virus at an early stage of the infection. However, one patient was positive by ELISA and negative by RT-PCR probably due to exposure to the virus without replication.

This significant change from negative to positive before and after treatment regarding HCV-PCR results can be related to repeated bone marrow aspirate and biopsy, repeated blood transfusions and/or hospitalization exposing patients of ALL to Nosocomial infection.

The poor sensitivity of anti-HCV assays to determine HCV infection in children with hematological disease is important to consider when such studies are being performed **[9]**. HBsAg was detected in 29.5% after treatment, compared to 4.5% before treatment. HBV DNA was detected in 9% by RT-PCR after 6 months of treatment, compared to negative results by RT-PCR in all patients before therapy. Four cases changed from negative to positive after treatment showing a highly significant change ($p=<0.001$).

As this study cohort included young age group, they were enrolled in the routine HBV vaccination within the national immunization program. Nevertheless, 4 cases were considered as currently infected cases with HBV.

In children with cancer vaccinated against HBV according to the vaccination schedule, the immune response maintains a protective level of anti-HBs in more than 60% of cases, despite immunosuppression. The protective level of HBsAb in patients treated for neoplastic diseases dropped from 80.5% at 3 months after end of vaccination program to 78.6% after 18 months of the end of the program **[10]**. It is clearly shown that children with previous vaccination had significantly lower rate of HBV infection than those receiving no primary vaccination. This finding strongly supports that HBV vaccine, as a part of routine vaccination before they develop ALL, is a key factor in decreasing the incidence of viral hepatitis in children with cancer **[11]**.

In a previous study, hospitalization was found to be the highest risk factor for both HCV and HBV infections in ALL pediatric patients, although HBV infection was controlled by a vaccine program that was initiated approximately 1 year earlier, where 18.6% of the children became infected with HCV **[9]**.

The risk of infection with both hepatitis B virus (HBV) and hepatitis C virus (HCV) is well documented in children with hematological disorders **[10]**. Many of these children receive multiple transfusions of different blood components, and this could be a potential risk factor for acquiring such infections. Also the children are highly immunosuppressed, and therefore the manifestations of these infections are mostly subclinical and rarely noticed **[11,12]**. Thus, HBV and HCV infections appeared often as "silent infections" in leukemic patients and were detected only if prevalence studies were performed or if the children underwent testing for HBV and HCV periodically as part of a routine procedure **[13]**.

In the current study, CMV- ELISA and RT-PCR results showed a change of 4.5%from negative to positive six months after treatment giving a high statistically significant change ($p$ value <0.001). EBV was positive by both ELISA and RT-PCR in 4.5% of patients after six months of treatment while one of them was already positive before treatment. EBV RT-PCR results showed change of one case (2.3%) from negative to positive after treatment giving rise to 2 positive cases (4.5%) instead of one (2.3%) before treatment. However, rate of change is statistically insignificant ($p$ value >0.05).

However, in both CMV and EBV testing, it was found that 42 patients were negative by both ELISA and RT-PCR and two patients were positive by both ELISA and RT-PCR, and there was no difference in results by the two tests (Kappa = 1, $p \leq 0.001$).

CMV IgG and EBV IgG detect past exposure and were done before and after treatment to detect reactive infection after treatment. New infections related to treatment procedures such as blood transfusion and hospitalization (nosocomial infection) which could only be proved if negative CMV IgG cases before treatment turned to be positive for CMV IgM by ELISA and CMV DNA positive by PCR or by increase in titre of the IgM antibody.

In the current study, positive CMV and EBV cases were previously infected suggesting that reactivation of the old dormant infection due to immunosuppression may be responsible rather than new infections related to therapy.

HHV8 was detected in the current study by Real time-PCR in 2.3% 6 months after treatment while it was negative before treatment. This has no significance and needs a study with a larger scale. Previous studies indicated that the presence of multiple HHVs, including EBV and CMV at high loads, is common in B-ALL and B-CLL cell samples **[2]**.

In the current study, all cases started chemotherapy shortly after establishment of diagnosis. Forty three cases received blood transfusion from 1 to 15 times (mean 7 times) during follow-up. Correlation between frequency of blood transfusion and positivity in CMV, EBV, and HHV8 could not be done due to small numbers of subgroups. On the other hand, positive HCV Ab results by ELISA, gave no sufficient correlation with the frequency of blood transfusion ($p$>0.05) while by RT-PCR, HCV infection gave high significant relation with increasing frequency of blood transfusion ($p$<0.005). This significant relation was not noted in the case of HBV even with the use of molecular diagnosis. This suggests that infection may be related to other diagnostic or therapy procedures as repeated sampling, BM aspirates and biopsies, chemotherapy or hospitalization.

A significant higher incidence of viral infections detected by molecular diagnosis after treatment, than before treatment, suggests that treatment by itself may be a source of co-infection which will add extra load aggravating the condition of ALL patients. The introduction of standardized nucleic acid identification reagents and kits has significantly reduced intra laboratory variability. The real time detection methods have reduced assay time to 2-3 hours and have significantly improved the ability to quantify viral nucleic acids **[14]**.

*Conclusion:*

In conclusion, pediatric ALL cases had a high significant prevalence of, treatment related, viral infections with HBV, HCV and CMV as

detected by real-time PCR. This indicates the necessity of initial screening as well as re-screening after therapy. The change from negative to positive result after therapy may be attributed to improve immune response or to contracting a new infection. Sources of infection may be related to repeated sampling, blood transfusion and Nosocomial infection.

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