ORIGINAL RESEARCH

Detection and Quantification of Epstein Barr Virus in Saliva and Subgingival Plaque in Paediatric Patients with Acute Lymphoblastic Leukemia.

Karthik Shunmugavelu[1], Kannan Ranganathan[2], Uma Devi K Rao[2], Elizabeth Joshua[2], Rooban Thavarajah[2]

Abstract

Background: Epstein Barr virus (EBV), an oncogenic human herpes virus, has been associated with malignant tumours of epithelial cells and B lymphocytes. Active replication in blood has been reported in 32% of Indian children with ALL by real time PCR. Epstein Barr virus is released into saliva from epithelial cells and saliva is known to play an important role in transmission of Epstein Barr virus. This study was designed to detect, quantify and assess the Epstein Barr virus in saliva and subgingival plaque of paediatric patients with acute lymphoblastic leukaemia and compare with controls. There are reports where EBV had been detected and quantified in saliva in hematopoietic malignancies other than ALL. There are no reported studies that have detected and quantified EBV in saliva and subgingival plaque samples.

Aim: To study the presence of Epstein Barr virus in saliva and subgingival plaque of paediatric patients with acute lymphoblastic leukaemia

Objective: To assess the prevalence of Epstein Barr virus in saliva and subgingival plaque of paediatric patients with acute lymphoblastic leukaemia using quantitative real time polymerase chain reaction.

Material and Methods: EBV DNA extraction was done from unstimulated saliva and subgingival plaque samples from healthy paediatric individuals (n=20) and paediatric patients with acute lymphoblastic leukaemia (n=20), as per the protocol of the Indigenous SmartPrepTM Genomic DNA Extraction Kit. The extracted DNA was subjected to quantitative real time Polymerase Chain Reaction (q-rtPCR) to detect and quantify EBV.

Results: Statistically significant difference was observed between ALL and controls with respect to age, height, weight, haemoglobin, red blood cell count, white blood cell count, platelet count and mean oral hygiene index. EBV was detected in 4 cases of saliva samples of ALL patients above the threshold value and all these 4 patients also presented with acute respiratory infections. There was a statistical significant difference between the two groups on comparison of threshold values (> 6.841) of final qPCR amplification plot (0.035). Mean EBV viral load in saliva samples in Group I (N=20) was 103812 ± 164452.2040 copies/ml and Group II (N=20) was 88464.0 ± 177418.3829 copies/ml. Statistically significant difference (p=0.004) was observed in the study groups with respect to EBV viral load.

Conclusion: EBV is present in some ALL patients and should be considered as a factor associated with systemic morbidity. Further studies are needed to ascertain a causal relationship, if any, between EBV and ALL in paediatric patients.

Keywords: Epstein barr virus, saliva, subgingival plaque, acute lymphoblastic leukaemia

Introduction

Epstein Barr virus was discovered by Epstein, Achong and Barr in 1964[1]. It is a double stranded deoxyribonucleic acid (DNA) virus categorized under the human herpes virus (HHV) family as HHV-4. EBV infects and immortalizes human B lymphocytes leading to Acute Lymphoblastic Leukemia (ALL), Burkitt's lymphoma, classic Hodgkin's lymphoma, post-transplant lymphoproliferative disorders, HIV-associated lymphoproliferative disorders (LPDs), primary central nervous system lymphoma, immunoblastic diffuse large B-cell lymphoma and plasmablastic lymphoma[2].

The Global Burden of Diseases, Injuries and Risk Factors Study (GBD) is an observational epidemiological study which describes the mortality and the morbidity for major diseases, injuries and risk factors to health at global, national and regional levels. GBD 2017 states that cancer among the paediatric population accounts for 2,40,942 cases of which 1,93,700 cases (80.3%) are hematopoietic malignancies of which 59,100 cases (3%) were Acute Lymphoblastic Leukae-

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How to cite the article: Karthik Shunmugavelu1, Kannan Ranganathan2, Uma Devi K Rao2, Elizabeth Joshua2, Rooban Thavarajah2. Detection and Quantification of Epstein Barr Virus in Saliva and Subgingival Plaque in Paediatric Patients with Acute Lymphoblastic Leukemia. Oral Maxillofac Pathol J 2024; 15(1). Page number. 10-18

Source of support: Nil

Conflict of interest: None

Acknowledgements: Dr Lavanya N, Dr Lavanya C, Dr Kavitha L, Dr Sudharshan, Dr Joseph, Dr Sarala Rajajee, Dr Ezhilarasi, Mrs Kavitha Wilson, Mr Rajan and Mrs Prescila Mary

mia (ALL)[3]. In India, GLOBOCAN (2017), a project of the International Agency for Research on Cancer (IARC) reports an annual incidence of 25,000 cases of pediatric cancer of which 9000 cases (36%) were hematopoietic malignancies with 3761 cases (41.7%) of ALL[4].

The role of EBV in the pathogenesis of ALL includes transformation of B cells through the EBV Latency III or growth program of viral expression for which Epstein Barr Nuclear Antigen Latent Protein (EBNA LP), EBNA 3C and EBNA 2 are viral gene products[5]. Seroprevalence of EBV antibodies among healthy children ranges from 20% to 80% 6. EBV infection in children generally occurs between the ages of 2 and 4 years and around 15 years. Below the age of one year, EBV infection is seen only in 50% of children[7]. The seroprevalence of EBV in paediatric population with ALL ranges from 8.3% to 83%[8,9,10]. EBV infection in ALL leads to c-myc oncogene activation and increased expression due to lymphocyte chromosome mutations or translocations, resulting in lymphoma[11]. ALL paediatric population with EBV seropositivity have poor prognosis[12].

As there is no evidence in literature reporting the detection of EBV in saliva and subgingival plaque in ALL patients, this study was done to detect and quantify EBV in saliva and subgingival plaque of paediatric population with ALL.

Materials and Methods

This cross sectional study was done in the Department of Oral and Maxillofacial Pathology, Ragas Dental College and Hospital and the Department of Paediatric Hemato- Oncology, Dr.Mehta's Hospital, Chetpet.

Study Groups:

Group I: Control group (n=20) healthy children,- saliva samples were taken in an unstimulated manner and subgingival plaque samples from each quadrant.

Group II: Study group (n=20) paedaitric ALL patients, confirmed by blood investigations and bone marrow aspirate – saliva samples were taken in an unstimulated manner and subgingival plaque samples from each quadrant.

The study groups (N=40) were stratified based on the EBV load into patients with EBV load ≤ 1000 copies /ml and > 1000 copies /ml of saliva[13].

Inclusion Criteria:

Group I:

- Less than 15 years healthy children

Group II:

- Less than 15 years ALL patients,
- ALL confirmed by blood investigations and bone marrow aspirate

Exclusion Criteria:

Paediatric patients with acute lymphoblastic leukaemia and normal category, not willing to give consent.

EBV DNA extraction was done from all saliva and subgingival plaque samples as per the protocol of the Indigenous SmartPrepTM Genomic DNA Extraction Kit. Taqman assay was performed on the Epstein-Barr virus target region U2 region encode Epstein-Barr nuclear antigen 2 (EBNA2) with an open Reading Frame Name (ORF) of BYRF1. The forward primer used was TTTGCTAGGGAGGAGACGTGTGT, reverse primer was AGACAACCACAGACACCGTCCT and CCGTCCCGGGTACAAGTCCCGGGTG with a 25 bases was used as the hydrolysis probe. The kit contained positive control template and PCR grade water (free of nucleases and nucleic acid contamination) was used as a negative control.

The data and descriptives were analysed by using SPSS software version 25 (Statistical Package for Social Sciences). All the numerical data were described as mean ± standard deviation. The significant association of the descriptive variables (gender, blood group, oral lesions, overall detection of EBV in study groups, detection of EBV in saliva samples and subgingival plaque samples of study groups) were analysed by using Chi-square test. Student T test was used to compare the significant difference between two means (age, height, weight, hemoglobin, red blood cell count, white blood cell count, platelet count, OHI index, DMFT index). P value < 0.05 was considered significant.

Results

Table 1: Distribution of age in the study groups (n=40)

	Group – I (n=20)	Group-II (n=20)	p Value
1-5 Years	0(0%)	14(70%)	**0.00****
6-10 Years	8(40%)	3 (15%)	
11-15 Years	12 (60%)	3 (15%)	

P<0.05**

Age was described in range and percentile (Table 1). In Group I (N=20), 8 cases (40%) were of 6-10 years and 12 cases (60%) were of 11-15 years. In Group II (N=20), 14 cases (70%) were of 1-5 years, 3 cases (15%) were of 6-10 years and 3 cases (15%) were of 11-15 years. There was a statistical significance observed between two groups on age comparison (p=0.00).

Table 2: Distribution of Gender In The Study Groups (N=40)

GENDER	GROUP I (n=20)	GROUP II (n=20)	p-Value
MALE	12 (60%)	12 (60%)	1.00
FEMALE	8 (40%)	8 (40%)	

On comparison of gender between the two groups(Table 2), there were 12 males (60%) and 8 females (40%) in Group I (N=20). There were 12 males (60%) and 8 females (40%) in Group II (N=20). There was no statistical significance between the two groups on gender comparison (p=1.00).

Table 3: Mean Height And Weight In The Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
HEIGHT (CMS)	121.80 ± 2.91	102.74 ± 1.51	**0.00****
WEIGHT (KGS)	21.92 ± 1.70	15.24 ± 4.37	**0.00****

p<0.05**

On comparison of height between two groups (Table 3), the mean height observed in Group I was 121.80 ± 2.91 (N=20) and in Group II was 102.74 ± 1.51 (N=20). There was a statistical significance observed between two on comparison of height (p=0.00). On comparison of weight between two groups, the mean weight observed in Group I was 21.92 ± 1.70 (N=20) and in Group II was 15.24 ± 4.37 (N=20). There was a statistical significance observed between two on comparison of weight (p=0.00).

Table 4: Distribution of blood grouping in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
A+	4(20%)	4(20%)	0.896
A-	2 (10%)	2(10%)	
B+	6 (30%)	7(35%)	
B-	1 (5%)	0(0%)	
O+	2 (10%)	1(5%)	
O-	0(0%)	0(0%)	
AB+	3 (15%)	5(25%)	
AB-	2 (10%)	1(5%)	

A +ve blood group was observed in 4 cases (20%) in both Group I (N=20) and II (N=20). A –ve blood group was observed in 2 cases (10%) in both Group I (N=20) and II (N=20). B + ve blood group was observed in 6 cases (30%) in Group I (N=20) and 7 cases (35%) in Group II (N=20). B –ve blood group was observed in 1 case (5%) in Group I (N=20) but none in Group II (N=20). O +ve blood group was observed in 2 cases (10%) in Group I (N=20) and 1 case (5%) in Group II (N=20). AB +ve blood group was observed in 3 cases (15%) in Group I (N=20) and 5 cases (25%) in Group II (N=20). AB –ve blood group was observed in 2 cases (10%) in Group I (N=2) and 1 case (5%) in Group II (N=20). There was no statistical significance observed in blood grouping among the two groups. (Table 4)

Table 5: Mean Hemoglobin levels in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
Hemoglobin g/dL	12.69 ± 1.25	8.60 ± 1.26	**0.00****

p<0.05**

In Group I (N=20), mean hemoglobin level was 12.69 ± 1.25. In Group II (N=20), mean hemoglobin level was 8.60 ± 1.26. A statistical significance was observed between the two groups on comparison of hemoglobin level (p=0.00). (Table 5)

Table 6: Mean RBC count in the study groups ((N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
RBC count per cubic mm	4.70 ± 0.47	2.87 ±0.48	**0.00****

p>0.05**

In Group I (N=20), the mean RBC count was 4.70 ± 0.47. In Group II (N=20), the mean RBC count was 2.87 ± 0.48. A statistical significance was observed between two groups on comparison of RBC count (p=0.00). (Table 6)

Table 7: Mean WBC Count in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
WBC COUNT Per cubic mm	8.47 ± 2.61	15.3 ± 2.69	**0.00****

P<0.05**

In Group I (N=20), the mean WBC count was 8.47 ± 2.61. In Group II (N=20), the mean WBC count was 15.3 ± 2.69. A statistical significance was observed between two groups on comparison of WBC count (p=0.00). (Table 7)

Table 8: Mean Platelet Level in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
PLATELET COUNT per cubic mm	3.14 ± 0.92	2.51 ± 0.48	**0.00****

P<0.05**

In Group I (N=20), the mean platelet count was 3.14 ± 0.92. In Group II (N=20), the mean platelet count was 2.51 ± 0.48. A statistical significance was observed between two groups on comparison of platelet count (p=0.00). (Table 8)

Table 9: Mean OHI index in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-value
OHI Index	0.75 ± 0.21	0.49 ± 0.43	**0.00****

P<0.05**

In Group I (N=20), the mean OHI was 0.75 ± 0.21. In Group II (N=20), the mean OHI was 0.49 ± 0.43. A statistical significance was observed between two groups on comparison of OHI (p=0.00). (Table 9)

Table 10: DMFT Index in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
DECAY	14 (70%)	8 (40%)	0.567
MISSING	3 (15%)	7 (35%)	
FILLED	3(15%)	5(25%)	

Decayed teeth were observed in 14 cases (70%) and 8 cases (40%) in Group I (N=20) and Group II (N=20) respectively. Missing teeth were observed in 3 cases (15%) in Group I (N=20) and 7 cases (35%) Group II (N=20). Filled teeth were observed in 3 cases (15%) in Group I (N=20) and 5 case (25%) in Group II (N=20). There was no statistical significance observed between two groups on comparison of DMFT (p=0.567). (Table 10)

Table 11: Distribution of Oral lesions in the Study Groups (N=40)

	GROUP I (n=20)	GROUP II (n=20)	p-Value
ASYMPTOMATIC	6 (30%)	0(0%)	**0.00****
ULCER	0(0%)	2(10%)	
GINGIVAL BLEEDING	0(0%)	8(40%)	
COATED TONGUE	0(0%)	1(5%)	
MUCOSITIS	0(0%)	0(0%)	
PALLOR	0(0%)	1(5%)	
DECAY	14(70%)	8 (40%)	

p<0.05**

In Group I (N=20), 6 cases (30%) were asymptomatic. Oral ulcer was observed in 2 cases (10%) in Group II (N=20). Gingival bleeding was observed in 8 cases (40%) in Group II (N=20). Coated tongue was observed in 1 case (5%) in Group II (N=20). Pallor was observed in 1 case (10%) in Group II (N=20). Decayed teeth were observed in 14 cases (70%) in Group I and 8 cases (40%) in Group II (N=20). There was statistical significance observed between two groups on comparison of oral lesions (p=0.00). (Table 11)

Table 12: Overall detection of EBV in both the samples of study groups (n=40)

	SALIVA (EBV)		SUBGINGIVAL PLAQUE (EBV)		p-Value
	D	ND	D	ND	
GROUP I (N=20)	9(45%)	11(55%)	9(45%)	11(55%)	**0.035****
GROUP II (N=20)	4(20%)	16(80%)	2(10%)	18(90%)	

P<0.05**

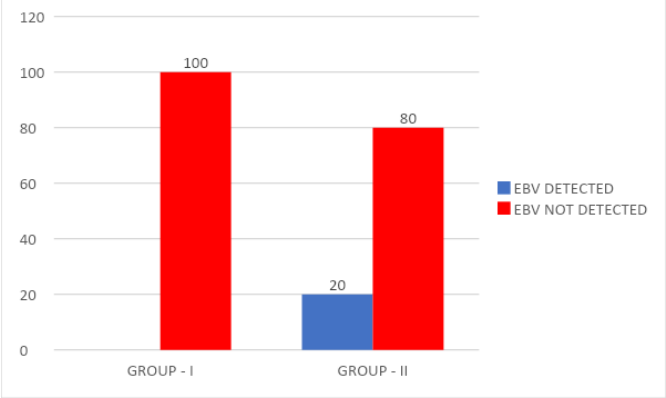
EBV was detected in saliva samples of 9 cases (45%) in Group I (N=20) and in 4 cases (20%) in Group II (N=20). EBV was not detected in saliva samples of 11 cases (55%) in Group I (N=20) and in 16 cases (80%) in Group II (N=20). EBV was detected in subgingival plaque samples of 9 cases (45%) in Group I (N=20) and in 2 cases (10%) in Group II (N=20). EBV was not detected in subgingival plaque samples of 11 cases (55%) in Group I (N=20) and in 18 cases (90%) in Group II (N=20). There was a statistical significance between the two groups on comparison of threshold values of final qPCR amplification plot (0.035). (Table 12)

Table 13: EBV detection in the saliva samples of the study groups (N=40) (Threshold Value = 6.841)

	GROUP – I (n=20)	GROUP – II (n=20)	p-Value
EBV DETECTED	0(0%)	4(20%)	**0.035****
EBV NOT DETECTED	20(100%)	16 (80%)	

P<0.05**

Graph 1: EBV detection in the saliva samples of the study group (N=40)

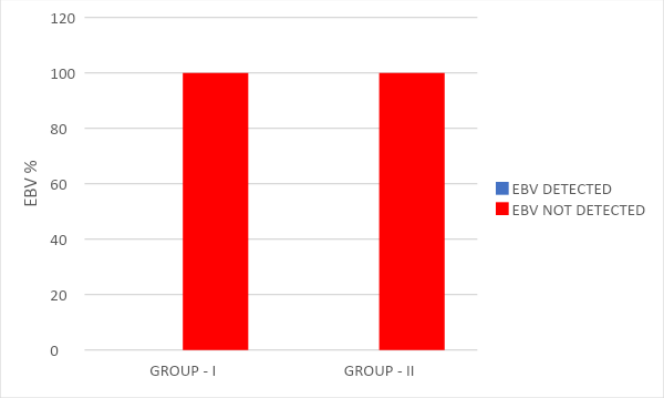


EBV was not detected in Group I (N=20) and but detected in 4 cases (20%) in Group II (N=20). EBV was not detected in 16 cases (80%) in Group II (N=20). There was a statistical significance between the two groups on comparison of threshold values (> 6.841) of final qPCR amplification plot (0.035). (Table 13 and Graph 1)

Table 14: Detection of EBV in the plaque samples of the study groups (N=40) *Threshold Value – 8.009

	GROUP – I (n=20)	GROUP-II (n=20)	P Value
EBV DETECTED	0(0%)	0(0%)	1.00
EBV NOT DETECTED	20(100%)	20 (100%)	

Graph 2: Detection of ebv in the plaque samples of the study groups (n=40)



EBV was not detected in subgingival plaque samples of Group I and Group II. There was no statistical significance between the two groups based on EBV subgingival plaque samples (p=1.00). (Table 14 and Graph 2)

Table 15: Detection of EBV in the saliva samples of the study groups (n=40) *Threshold Value not considered

	GROUP – I (n=20)	GROUP – II (n=20)	p-Value
EBV DETECTED	9(45%)	4(20%)	0.507
EBV NOT DETECTED	11(55%)	16 (80%)	

EBV was detected in Group I (N=20) of 9 cases (40%) and 4 cases (20%) in Group II (N=20). EBV was not detected in Group I (N=20) of 11 cases (55%) and 16 cases (80%) in Group II (N=20). There was no statistical significance between the two groups based on EBV in saliva samples (p=0.507). (Table 15)

Table 16: Detection of overall EBV in the Plaque samples of the study groups (N=40)

*Threshold Value not considered

EBV DETECTION	GROUP – I (n=20)	GROUP–II (n=20)	p-Value
EBV DETECTED	9(45%)	2(10%)	0.197
EBV NOT DETECTED	11(55%)	18 (90%)	

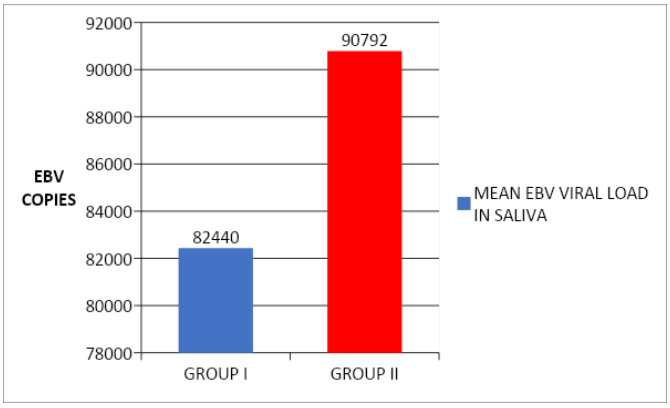
***Threshold Value not considered**

EBV was detected in Group I (N=20) of 9 cases (45%) and 2 cases (10%) in Group II (N=20). EBV was not detected in Group I (N=20) of 11 cases (55%) and 18 cases (90%) in Group II (N=20). There was no statistical significance between the two groups based on EBV in saliva samples (p=0.197). (Table 16)

Graph 3: EBV Viral Load in Saliva

	GROUP - I	GROUP-II	P VAL-UE
EBV SALIVA	82440±164452	90792±177418.3829	**0.004****

P<0.05**



Total number of copies for EBV viral load in control saliva samples was 1648806 copies. Mean EBV viral load in saliva samples of controls was 82440. Total number of copies for EBV viral load in ALL saliva samples was 1815840 copies. Mean EBV viral load in saliva samples of ALL patients was 90792. There was a statistical significant difference between the saliva samples of controls and ALL patients in the mean EBV viral load(p=0.004)(Graph 3)

Graph 4: EBV Viral load in Subgingival Plaque

	GROUP - I	GROUP-II	P VALUE
EBV PLAQUE	79355± 129351.4898	20690± 129351.4898	**0.009****

P<0.05**

Total number of copies for EBV in control subgingival plaque samples was 1587114 copies. Mean EBV viral load in subgingival plaque of controls was 79355. Total number of copies for EBV in subgingival plaque samples of ALL patients was 413802. Mean EBV viral load in subgingival plaque of ALL was 20690. There was a significant difference between the subgingival samples of controls and ALL patients in the mean EBV viral load(p=0.009) (Graph 4)

Discussion

EBV is a double stranded deoxyribonucleic acid (DNA) virus categorized under the human herpes virus (HHV) family as HHV-4. EBV infects and immortalizes human B lymphocytes leading to Acute Lymphoblastic Leukemia (ALL), Burkitt's lymphoma, classic Hodgkin's lymphoma, post-transplant lymphoproliferative disorders, HIV-associated lymphoproliferative disorders, primary central nervous system lymphoma, immunoblastic diffuse large B-cell lymphoma, HHV-8–positive primary effusion lymphoma and its solid variant plasmablastic lymphoma (LPDs)[14].

Guan et al in 2017 from the Department of Hematology, Qingdao University Medical College, Qingdao, China investigated the relationship between EBV infection and immunophenotyping of acute lymphoblastic leukemia (ALL) and chromosome aberrations and detected EBV-DNA copy numbers from bone marrow in 110 cases of patients with ALL, 75 cases of patients with acute myeloid leukemia (AML), and 37 cases of hematologically healthy control subjects by using Fluorescence quantitative polymerase chain reaction (FQ-PCR). In their study, EBV positivity in the ALL and AML groups was higher than in healthy subjects ($P < 0.05$). Also EBV positivity in B-ALLs increased significantly compared to the rate of T-ALLs ($P < 0.05$). EBV+ -ALLs reported with higher relapse and mortality rates than that of EBV- -ALLs emphasizing the fact that the infection rate of EBV in B-ALL was higher than that of T-ALL with an unfavorable prognosis[15]. Till date, there are no studies which have been done to detect EBV in saliva and subgingival plaque samples of ALL patients. In the present study, an attempt was made to detect and quantify EBV in saliva and subgingival plaque in children presenting with ALL and in the control group, comprising of healthy children.

In the present study, we observed that 85% of cases in ALL were in the age group below 10 years of which 70% were in the age group of 1-5 years. Due to this pattern of age distribution among ALL patients, there was a significant difference between the controls and ALL patients (p=0.00). Our results are in agreement with previous studies. Foa et al in 2011 from the Division of Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University, Rome, Italy stated that there is a peak occurrence of ALL in the age groups 1–5 and 5–10[16].

In present study, we observed that there was no significant difference in gender among the groups. Khalid et al in 2010 from Royal Darwin Hospital, Casuarina, Australia reported that ALL was more prevalent in males as compared to females. This difference in gender distribution could be due to a different study setting[17].

The mean height in centimetres among ALL patients in our cohort was 102.3 ±1. 5 and it was 122 ± 3 in controls and this difference was significant (p=0.00). The mean weight in ALL was 15.24±4.37 and 21.92±1.70 in controls. We found a decrease in the mean weight which had a statistical significance (p=0.00) between ALL patients and controls. Thus in this study, the mean height and weight were lower in the ALL Group than in the control group, which could probably be due to the influence of systemic compromise observed in ALL Patients. In contrast Browne et al in 2018 from the Department of Pediatric Medicine, St. Jude Children's Research Hospital, Memphis, Tennessee reported that there was an increase in the weight and decline in height in children with ALL patients. The difference could probably be, due to the study setting, age of diagnosis and influence of systemic compromise in ALL patients[18].

In the present study, among ALL patients, B+ (35%) was the most frequently seen blood group followed by AB+ (25%), A+ (20%) and A-(10%). Blood groups B- and O- were not present and AB- was seen in only one case. In controls, B+ (30%) was the most frequently seen blood group followed by A+ (20%), AB+ (15%), A-(10%), O+(10%), AB-(10%) and B-(5%). Blood group O- was not present. There was no significant difference in the pattern of distribution of blood groups between ALL patients and controls which is consistent with the literature. Ghali et al in 2017 from Department of pediatrics, College of Medicine, Baghdad University observed the data of blood group distribution in ALL patients and reported that there was no significant difference in the pattern of distribution between the normal individuals and those with ALL[19].

In the present study a significant difference was observed on comparison of hemoglobin level between ALL and controls (ALL - 8 ±1.26 gm/dl, controls = 12 ±1.25gm/dl) (p = 0.00). Hann et al in 1981 from the Department of Haematology and Department of Oncology, Royal Manchester Children's Hospital reported that ALL patients with low hemoglobin levels had better prognosis due to pretreatment proliferative activity of bone marrow blast cells and added that, children with ALL, who have high WBC and Hb levels have a rapidly multiplying malignancy, and they present with full blown ALL before the Hb has time to lower than in controls[20]. Our findings are in agreement with their study. In our study, the mean red blood cell count in the ALL patients was 2.87±0.48 and in the controls it was 4.70±0.47. This difference was statistically significant (p=0.00).

The mean white blood cell count in the ALL patients were significantly higher compared to the controls and this difference was statistically significant and consistent with the literature (ALL – 15.3±2,69, controls = 8.5 ±2.6) (p = 0.00). Hann et al in 1981 stated that high white blood cell count at the time of diagnosis in children with ALL was associated with better prognosis82. The mean platelet count in the ALL patients was 2.51±0.48 and in the controls it was 3.14±0.92. This difference was statistically significant (p=0.00). Bhushan et al in 2017 stated that ALL patients presented with normal platelet count in comparison to healthy children, and this difference could be due to different study setting and timing of recording of the parameters[21].

We observed a difference in the mean OHI between controls (0.75±0.21) and ALL patients in the present study (0.49±0.43) (p=0.00). Kapoor et al in 2019 from Department of Pediatric Hematology and Oncology, Rajiv Gandhi Cancer Institute and Research Centre, New Delhi, India reported that children with ALL undergoing treatment and following an oral care protocol presented with a good oral health status when compared with healthy children who did not follow a prescribed protocol[22]. Our finding could be attributed to the quality of oral hygiene protocol being followed in our study group.

There was no difference in the mean DMFT index (p=0.567) between the controls and ALL patients. Bica et al in 2017 from Department of Pediatric Dentistry and Orthodontics, University of Medicine and Pharmacy, TîrguMureş, Romania reported that there was no difference in the occurrence of decayed teeth between the controls and ALL patients[23,24].

In this present study, we observed a difference in the occurrence of oral lesions between ALL patients and the control groups. In ALL patients, the predominant oral manifestation was gingival bleeding (40%) followed by decayed teeth (40%), ulceration (10%), coated tongue (5%) and pallor (5%) (p=0.00)[25,26].

EBV was detected in blood samples of patients with ALL, rheumatoid arthritis, Hodgkin's lymphoma (HL), non-Hodgkin's lymphoma (NHL), hemophagocytic lymphohistiocytosis

(HLH), and in post transplantation lymphoproliferative disorder (PTLD), as well as in solid tumors such as in undifferentiated nasopharyngeal carcinoma (NPC), multiple sclerosis, infectious mononucleosis, B cell / T cell / Natural Killer cell lymphoproliferative disorders and B cell lymphomas by using qPCR. EBV has also been detected in saliva of patients with sickle cell disease, in HIV patients with and without hairy leukoplakia, infectious mononucleosis, in solid tumours after allogeneic haematopoietic stem cell therapy and in patients with multiple sclerosis by using qPCR[15].

Subgingival plaque EBV has been demonstrated in patients diagnosed with periodontitis and periimplantitis by using qPCR. Till now there are no studies in which saliva and subgingival plaque have been used to detect and quantify EBV in children with ALL.

In the present study, a total of 40 cases were included to detect EBV in saliva and subgingival plaque samples (controls=20, ALL=20). In the saliva samples, EBV was detected in 45 % of the controls and 20% of ALL patients. In subgingival plaque EBV was detected in 45% of controls and in 20% of ALL patients[27,28,29,30].

All the samples of saliva and plaque submitted for real time PCR analysis were considered as EBV positive when a sample exhibits a growth curve for EBV within 38 cycles. EBV negativity is indicated if the growth curve does not cross the threshold line within 38 cycles. The estimated threshold value was 6.481 copies/µl for saliva samples and 8.009 copies/µl for subgingival plaque samples. The viral load of each sample was determined and the mean viral load was calculated for both salivary and subgingival plaques. Above the threshold value of 6.841, EBV was detected in 20% (4 patients) of ALL patients, resulting in a significant difference (p=0.035). Mean EBV viral load in saliva samples of controls was 82440±164452 copies/ µl. Mean EBV viral load in saliva samples of ALL patients was 90792± 177418.3829/ µl. We observed a significant difference in the mean EBV viral load in the saliva samples of controls and ALL patients (p=0.004). Mean EBV viral load in subgingival plaque of controls was 79355± 129351.4898 copies/ µl. Mean EBV viral load in subgingival plaque of ALL was 20690± 129351.4898 copies/ µl. There was a significant difference in the mean EBV viral load in subgingival plaque samples of controls and ALL patients (p=0.009). Though EBV was detected in subgingival plaque in controls and ALL patients, none of the cases had a value of greater than threshold value of 8.009 and this could be probably due to the variable nature of subgingival plaque and the poor reservoir status[31,32].

An interesting observation in this study was that the most common variable among the 4 cases in whom EBV was detected and crossed the (saliva samples of ALL patients) threshold value above 6.841 had acute respiratory infections[33].

Imbronito et al in 2008 from the Department of Periodontology, Dental School, University of Sao Paulo, Brazil reported EBV positivity in subgingival plaque, saliva and peripheral blood as 45%, 37.5% and 25% respectively[34,35,36]. Sehgal et al in 2010 from the Department of Immunopathology, Pediatrics, Postgraduate Institute of Medical Education and Research, Chandigarh, India studied the association of EBV in childhood leukemia and concluded that EBV positivity was seen in 8/25 patients of ALL accounting for 32%[37,38].

Sehgal et al in 2010 reported causal relationship between EBV and lymphoreticular malignancies[37]. Wolf et al in 1990 from the Department of Pathology, Tufts University School of Medicine, Boston, Massachusetts reported that 4 out of 6 cases of hairy cell leukaemia indicate EBV oncogenic potential[39]. Sehgal et al in 2010 from the Department of Immunopathology, Pediatrics, Postgraduate Institute of Medical Education and Research, Chandigarh, India stated that in EBV immunoglobulin (Ig) G positive mothers with EBV Ig M positivity was observed[37]. In these mothers, younger siblings were protected from the risk of malignancies being caused due to such EBV infections. Their study also reported that one third of cases of childhood leukaemia present with EBV infection. In India, EBV might be an important coinfection in ALL patients and also could be an increased risk of ALL in offsprings if the mother is positive. EBV presence in children, may itself not cause ALL, but could synergistically cause an opportunistic infection, which would contribute to the onset of ALL.

From the results of present study, we observed that though EBV was detected in saliva and subgingival plaque samples of paediatric patients with acute lymphoblastic leukaemia, it crossed the threshold levels only in 20% of ALL patients. ALL children were recruited in the study immediately after the initial diagnosis, before starting chemotherapy. Thus chemotherapy could not have been a confounding factor for not detecting EBV. Based on this study results, we could state that salivary samples could be used to detect and quantify EBV but not subgingival plaque samples due to its biofilm nature. Further studies need to be carried out with respect to EBV detection and quantification in saliva and subgingival plaque.

There are studies which have reported that EBV positivity in ALL patients was associated with an unfavourable prognosis [40]. It has also been stated that EBV could be considered as a prototype of oncogenic viruses, which play the role of transforming agents and the resulting expression of viral RNAs and proteins could lead to induction of transformed phenotype. On this basis and strict association with distinct tumour types, EBV has been classified as Group 1 carcinogen.

In the context of children with ALL, it has been stated that[10] EBV reactivation during pregnancy may be a surrogate marker for some environmental exposures that could predispose to childhood leukaemia. There are studies which have identified a link between maternal EBV reactivation and development of ALL in the offspring. Given the evidence in the literature that EBV associated in children could be linked to maternal EBV activation and development of ALL in the offspring and the associated unfavourable prognosis of these children, screening of children with ALL for EBV could be essential to understand the prognosis of ALL and to identify this subgroup of patients.

Conclusion

This study was done to detect and quantify EBV in the saliva and subgingival plaque of paediatric patients with acute lymphoblastic leukaemia (ALL). 20 ALL patients and 20 healthy children were included in the study. DNA extraction was done from all samples using Indigenous SmartPrepTM Genomic DNA Extraction Kit and q-PCR was done using Indigenous SmartDiaTM EBV Real-Time PCR Kit. ALL was observed more in the age group 1 – 5 years in comparison to controls (p=0.00). Decrease in mean height was observed in ALL patients com-

pared to controls (p=0.00).

Decrease in mean weight was observed in ALL patients compared to controls (p=0.00).

Decrease in hemoglobin level and RBC count was observed in ALL patients compared to controls (p=0.00). Increase in WBC count was observed in ALL patients compared to controls (p=0.00).

Decrease in platelet count was observed in ALL patients compared to controls (p=0.00). Decrease in mean OHI index was observed in ALL patients compared to controls (p=0.00). ALL patients presented with increased gingival bleeding and decayed teeth compared to controls. EBV was detected in 20% in saliva samples and 10% in subgingival plaque samples in ALL patients. But it crossed the threshold values of 6.841 in 4 patients in saliva samples but none in subgingival plaque samples (the threshold value was 8.009). Those patients in whom EBV positivity was present, acute respiratory infection was also seen. Increased mean EBV viral load was observed in saliva samples of ALL patients compared to controls (p=0.004). Our study showed that saliva harbors EBV and studies are needed to further understand their role.

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