Epstein-Barr Virus Infection in Kenyan Children with Tonsillar Enlargement

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Abstract

Background: Enlarged tonsils and adenoids are common in children, but excessive growth can obstruct the airways. The tonsil, as a secondary lymphoid organ, harbours various pathogens, including Epstein-Barr virus (EBV), a widespread human herpesvirus that establishes lifelong latency in B cells, often commencing in the tonsils. Tonsil size typically decreases with age, with younger children having larger tonsils. However, the influence of EBV on tonsillar hypertrophy in malaria-endemic regions remains unclear.

Materials and methods: In this cross-sectional study, data were collected from 102 children aged between one and 14 years undergoing routine tonsillectomy. Finger-prick blood was collected before tonsillectomy, while tonsil tissue was collected during the surgery and transported on ice for analysis. Clinical and demographic data were recorded. EBV loads in tonsils and *Plasmodium falciparum (pf)* parasitemia were determined by quantitative polymerase chain reaction (qPCR). Based on the EBV status, Children were categorised into two groups and the demographic as well as clinical parameters were compared between the groups.

Results: EBV was detected in 58.1% of the participants, while 17.6% tested positive for malaria infection by qPCR. Adenotonsillar hypertrophy accounted for 44.1% of tonsillectomy cases. EBV-positive children had significantly lower haemoglobin levels as compared to EBV-negative individuals (p=0.04). Tonsil size showed a positive correlation with age (p = 0.037) and was significantly larger in malaria-negative children compared to malaria-positive (p<0.001).

Conclusion and Recommendation: The higher proportion of malaria-negative children who were EBV positive suggests a possible relationship between repeated malaria infections and EBV detection. Lower haemoglobin levels observed among EBV-positive children suggest that EBV infection may influence haemoglobin levels. Further research is needed to better understand these relationships.

Keywords: *Tonsils, Tonsillar enlargement, malaria exposure, Epstein-Barr Virus*

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Introduction

Tonsils are secondary lymphoid organs located within the pharynx and play a critical role in immunity as the first line of defence against infections(1). Tonsils undergo both morphological and B-cell phenotype changes with age (2,3). Typically, children have the largest tonsils due to frequent exposure to various infections, which can cause tonsillar enlargement (tonsillar hypertrophy) (4). Although it is normal for young children to have enlarged tonsils and adenoids, they can contribute to respiratory distress if they grow very large to block the airway (5). While tonsils are essential for immune defence, they serve as a critical site for Epstein-Barr virus (EBV) persistence (1).

Epstein-Barr virus (EBV) is a common human herpesvirus that establishes lifelong latent infections in B cells and is associated with various malignancies, including Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric

carcinoma and endemic Burkitt's lymphoma (eBL) (6–9). Notably, eBL is one of the most prevalent childhood cancers in sub-Saharan Africa, where malaria infection is endemic (10,11). The interplay between malaria and EBV is essential in the development of eBL, with studies showing that early infection with EBV in children from malaria endemic areas significantly increases the risk of eBL (12,13). Waldeyer's ring, which includes the tonsils, serves as a reservoir of EBV-infected B and T cells, exhibiting both latent and lytic viral replication (14–17). However, the role of EBV in tonsillar enlargement, particularly in malaria high-risk regions like sub-Saharan Africa, remains unclear.

Tonsillar hypertrophy is the enlargement of the tonsils beyond their normal size, causing symptoms such as difficulty breathing, obstructive sleep apnea, hypersomnolence, and may also lead to cardiac and respiratory complications (18). Tonsil enlargement may necessitate the removal of the tonsil tissue, a procedure referred to as tonsillectomy (19,20). The main clinical indications for tonsillectomy are: obstructive sleep apnea, recurrent tonsillitis, adenotonsillar hypertrophy, recurrent peritonsillar abscess and adenoid hypertrophy (21–25). To determine the size of the tonsils, the Brodsky scale is usually used as a standard system for grading tonsil size in clinical oropharyngeal investigation (26). This scale is based on how much of the oropharyngeal airway the tonsils occupy in the medial-lateral dimension(27). Tonsils are graded as follows: grade 0 (in the fossa), grade 1 (occupying ≤25% of the oropharyngeal width), grade 2 (26-50% of oropharyngeal width), grade 3 (51-75% of oropharyngeal width) and grade 4 (more than 75% of oropharyngeal width). These grading scales are important in recording and communicating changes in tonsil enlargement.

Tonsillar hypertrophy may contribute to viral transmission. In a prior study of patients with infectious mononucleosis, children with tonsillar enlargement shed higher levels of Epstein–Barr virus (EBV) in saliva than those without enlargement, with salivary EBV loads closely mirroring tonsillar viral loads and declining progressively following tonsillectomy(28). Apart from EBV DNA, serum levels of viral capsid antigen (VCA) and Epstein-Barr virus nuclear antigen (EBNA) were significantly higher in patients with grade 3-4 tonsillar hypertrophy as compared to patients with grade 1-2. Additionally, the frequency of grade 3-4 was higher among EBV-positive individuals than among EBV-negative individuals (29). Together, these observations collectively suggest a positive correlation between EBV infection and tonsillar size, highlighting the potential role of tonsillar enlargement in EBV transmission and disease progression. However, the influence of EBV infection on tonsillar enlargement in children living in malaria-endemic regions remains unclear.

This study, therefore, aimed to address the following questions: first, to detect EBV in the tonsils of children; and second, to evaluate and correlate the clinical and laboratory features of paediatric patients with tonsillar hypertrophy in relation to EBV infection.

Materials and Methods

Study design and population

This cross-sectional study recruited 102 children undergoing tonsillectomy at Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH). This hospital is situated in a high malaria transmission region of western Kenya.

The sample size was determined using standard prevalence study calculations with the Scalex and ScalaR calculators, as described by Naing *et al*., 2022.

All children aged 1 to 14 years who tested negative for malaria by both blood smear and rapid diagnostic test (RDT) (SD-Bio line, Korea), and who had haemoglobin levels above 7 g/dL, as measured by the Hemocue machine (Hemocue AB, Sweden), were considered eligible to participate in the study. All children with haemoglobin levels lower than 7 g/dL or had chronic conditions such as HIV or diabetes, as documented in hospital records, were excluded from participating in the study.

Sample collection, processing and cryopreservation

Finger-prick blood was collected as previously described (30). Surgically removed tonsils were graded as grade 1-4 based on Brodsky scale(26), Placed into a 50 ml centrifuge tube with ice-cold sterile hanks balanced salt solution (HBSS) containing 5% fetal bovine serum (FBS), 10 mM Glutamine, 0.05 mg/ml Gentamicin and 1% Antibiotic-Antimycotic mix (Penicillin, Streptomycin and Amphotericin B) and transported to the laboratory within two hours.

The tonsil tissue was weighed, placed on a 60 mm plastic petri dish on ice, and moistened with HBSS. Blood clots, fat and connective tissues were removed, and the tonsil tissue was cut into 3-10 mm fragments and filtered through a 70 µm cell strainer placed on top of a 50 ml centrifuge tube, as demonstrated previously (3).

Tonsillar mononuclear cells (TMC) were then isolated from the cell suspension over Ficoll-Hypaque as described earlier (3). The total number of mononuclear cells was determined using a hemocytometer and cryopreserved in freezing medium (70% RPMI 1640, 20% FBS and 10% DMSO) in liquid nitrogen until analysis.

DNA extraction and quantitative PCR detection of *Plasmodium falciparum*

200µl of finger-prick blood was used to extract DNA using the DNA mini kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions as described earlier (31). *P. falciparum* parasitemia levels were then determined using qPCR on the Quant Studio 6 Flex (Applied Biosystem).

qPCR experiments were run targeting the *pf* 18S ribosomal gene. Primers sequences are tabulated in Supplementary Table 1. The program included: 50ºc for 2 min, followed by 95ºc for 3 mins, followed by 95ºc for 10 seconds, then 60ºc for 30 seconds, and finally go to step 3, 40 times. Using a standard curve, the parasitemia values were generated and exported to an Excel file.

DNA extraction and quantitative PCR detection of EBV and EBV typing from tonsils

Previously frozen ($I\times10^7$ cells) Tonsillar mononuclear cells (TMCs) were used to extract DNA using the DNA mini kit (Qiagen, Dusseldorf, Germany) as per the manufacturer's instructions, as previously described (3). Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) was used to assess the concentration of the DNA.

Epstein-Barr virus was then detected by qPCR using Quant Studio 6 flex (Applied Biosystem) using Primers and probes, targeting a 70 bp region of EBV BALF5 as previously described (12,31). The program was as follows: 50ºc for 2 min, followed by 95ºc for 2min, followed by 95ºc for 0.01 seconds and 60ºc for 20 seconds, finally go to step3 40 times. A standard curve was generated from a plasmid (generated from the BL cell line), which contains 2 copies of the EBV genome in each cell. A standard curve was used to extrapolate values for each specific sample run. β–actin was used as a positive control.

Epstein-Barr virus typing was performed as described earlier (32). Briefly, this was done by targeting the EBNA3C gene. Probes were used to differentiate between EBV type 1 and type 2. The program included: 50ºc for 2 min, followed by 95ºc for 2 mins, followed by 95ºc for 0.01 seconds, then 60ºc for 20 seconds, and finally go to step 3, 40 times. All primer sequences are tabulated in Supplementary Table 1.

Statistical analysis

All statistical analyses were performed using R (version 4.3.1) software. Descriptive statistics were calculated using Fisher's exact tests for categorical variables, while the Mann-Whitney U test was employed to assess the relationship between clinical and laboratory characteristics of study participants based on EBV status. Log-transformed EBV loads and tissue sizes were assessed using linear regression.

A P-value of <0.05 at 95% Confidence interval was considered significant.

Ethical approval

Ethical approval of this study was obtained from Kenya Medical Research Institute (KEMRI) Scientific and Ethical Review Unit (SERU) (protocol No: 4527) and the Institutional Scientific Ethics Review Committee (ISERC) of Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH). Written informed consent was obtained from parents/guardians, and assent was obtained from children over 7 years of age.

Results

The process of sample collection and analysis is illustrated in Supplementary Figure 1. The majority of the study participants were males (n =76) (74.5%), and the median age of the study participants was 3.8 (2.6- 5.5) years. Detailed clinical and demographic information is represented in Table 1.

Frequency of clinical indications for tonsillectomy

The clinical indication observed among the study population showed that adenotonsillar hypertrophy was the main indication for tonsillectomy (44.1%), followed by grade 1-4 tonsils (31.4%), adenoid hypertrophy (5%) and other conditions, including nasal blockage, breathing difficulties, adenoids and recurrent tonsillitis (19.6%) (Table 2).

Increased EBV detection in malaria-negative children

We previously reported the detection of EBV to be common among children with malaria (31). Although all participants in this study tested negative for malaria at enrolment, 18 out of 85 individuals had subclinical malaria. Among EBV-positive individuals, 16% were infected with malaria. In contrast, EBV negativity was observed in 69.4% of individuals who tested negative for malaria compared with 27.8% of individuals who tested positive for malaria ($p < 0.001$; Table 3). In addition, a borderline but statistically significant difference in body temperature was noted: EBV-positive participants had a lower median temperature (36.5°C, IQR 36.1–36.7) than EBV-negative participants (36.8°C, IQR 36.4–36.9; $p = 0.045$; Table 3).

Haemoglobin levels observed among EBV-positive individuals

Evaluation of the effect of EBV infection on clinical indicators revealed that EBV-positive individuals had lower haemoglobin levels (Median 11.2g/dL, IQR 10.7-12.1) compared to EBV-negative individuals (median 12.2g/dL, IQR 11.4-12.9; $p = 0.004$) (Table 3).

Association between clinical and demographic factors and EBV loads

Assessment of the association between clinical, demographic and EBV viral loads showed no significant relationship (Table 4).

Table 1

Clinical and Demographic Characteristics of the study Participants (N = 102)

Variable		n (%)
Age (years)	Median (IQR)	3.8 (2.6-5.5)
Sex	Male	76 (74.5%)
	Female	26 (25.5)
Nutritional status	Well-nourished	93 (91.2%)
	Malnourished	9 (8.8%)
Malaria status	Positive	18 (17.6%)
	Negative	67 (65.7%)
Body temperature (°C)	Median (IQR)	36.5 (36.2-36.9)
Haemoglobin (g/dL)	Median (IQR)	11.8 (10.8-12.6)
EBV loads (copies/µg)	Median (IQR)	4371 (1908.6-9751.6)
Size of tissue (mg)	Median (IQR)	1200 (960-1650)

Note. Body mass index for age as an indicator for nutritional status; malnourished <= -2 Z scores, Well-nourished > -2 Z scores. Malaria status of participants as determined by quantitative PCR (qPCR)

Association between age, malaria status and tonsil tissue size

The association between clinical, demographic characteristics and tissue size revealed that older children had significantly larger tissue size compared to younger children (3.01, 95% CI 2.84-3.19), P =0.037). Additionally, tissue size was larger among children who tested negative for malaria (3.07, 95% CI 2.65-3.48) compared to those who tested positive for malaria (3.06,95% CI 2.69-3.45) (p<0.001) (Table 5).

Changes in tissue size and EBV DNA loads do not affect Brodsky grades

Assessment of the distribution of tissue size across the Brodsky grades did not significantly influence grading (p-value= 0.983) (Figure 1a).

Table 2

Clinical Indications for Performing Tonsillectomy (N= 102)

Reason for Tonsillectomy	n (%)
Adenotonsillar hypertrophy	45 (44.1%)
Adenoid hypertrophy	5 (4.9%)
Grade 1-4 tonsils	32 (31.4%)
Other	20 (19.6%)

Table 3

Clinical and Laboratory Characteristics of Study Participants Based on EBV Status (N = 86)

Variable		EBV+	EBV -	p value
Age (years)	Median (IQR)	3.5 (2.4-5)	4.6 (3-6.5)	0.061
Sex	Male	35 (70%)	28 (77.8%)	
	Female	15 (30%)	8 (22.2%)	0.614
Nutritional status	Well-nourished	46 (92%)	34 (94.4%)	
	Malnourished	4 (8%)	2 (5.6%)	0.310
Malaria status	Positive	8 (16%)	10 (27.8%)	
	Negative	42 (84%)	25 (69.4%)	<0.001
Body temperature (°C)	Median (IQR)	36.5 (36.1-36.7)	36.8 (36.4-36.9)	0.045
Haemoglobin (g/dL)	Median (IQR)	11.2 (10.7-12.1)	12.2 (11.4-12.9)	0.004
Size of tissue (mg)	Median (IQR)	1057.5 (900-1475)	1300 (788-1525)	0.351

Note. Body mass index for age as an indicator for nutritional status; malnourished <= -2 Z scores, Well-nourished > -2 Z scores; Fisher's test for proportions and Mann-Whitney test for means.

Table 4

Association between Clinical and Demographic Characteristics with EBV Viral Load (N = 102)

Variable	Estimate (95% CI)	p value
Age (years)	3.75 (3.32-4.18)	0.492
Sex		
Male	3.74 (3.54-3.93)	
Female	3.75 (2.89-4.07)	0.211
Nutritional status		
Well-nourished	3.69 (2.50-4.88)	
Malnourished	3.47 (2.89-4.05)	0.470
Malaria status		
Positive	3.66 (3.48-3.84)	
Negative	3.72 (3.09-4.36)	0.786
Body temperature (°C)	11.82 (3.00-26.63)	0.249
Haemoglobin (g/dL)	4.33 (2.50-6.15)	0.401
Size of tissue (mg)	3.77 (3.51-4.04)	0.083

Note. Body mass index for age as an indicator for nutritional status; malnourished <= -2 Z scores, Well-nourished > -2 Z scores; linear regression analysis. Malaria status of subjects as determined by quantitative PCR (qPCR).

Finally, EBV loads were compared across Brodsky grades among individuals with a complete data set (n=15), showing no difference in distribution across the grades (Figure 1b).

Discussion

Tonsils are key immune organs in the pharynx (1), and are usually large in children due to frequent infections (4). However, excessive growth can lead to tonsillar enlargement, causing abnormalities such as difficulty in breathing, obstructive sleep apnea, hypersomnolence and even cardiac and respiratory complications (18). In severe cases, tonsillar enlargement may require tonsillectomy (19,20).

While tonsils are essential for immune defence, they serve as a critical EBV reservoir, harbouring infected B cells that can differentiate into immortalised memory B cells, facilitating persistent infection (14). EBV infection causes enlargement of tonsils by transforming memory B cells into blast cells, leading to persistent proliferation (33,34).

Tonsillectomy remains the main procedure for children with tonsillar enlargement. Previously, the main indication for surgery in the paediatric population was recurrent throat infection diagnosed with group A beta- haemolytic streptococcal infections (GABHS) (35,36).

Table 5

Association between Clinical and Demographic Characteristics with Tissue Size (N = 102

Variable	Estimate (95% CI)	p value
Age (years)	3.01 (2.84-3.19)	0.037
Sex		
Male	3.11 (3.03-3.20)	
Female	3.16 (2.91-3.40)	0.589
Nutritional status		
Well-nourished	3.12 (2.61-3.63)	
Malnourished	3.21 (3.00-3.46)	0.449
Malaria status		
Positive	3.06 (2.69-3.45)	
Negative	3.07 (2.65-3.48)	<0.001
Body temperature (°C)	3.19 (2.15-8.54)	0.979
Haemoglobin (g/dL)	3.13 (2.39-3.86)	0.998

Note. Body mass index for age as an indicator for nutritional status; malnourished <= -2 Z scores, Well-nourished > -2 Z scores; linear regression analysis. Malaria status of subjects as determined by quantitative PCR (qPCR).

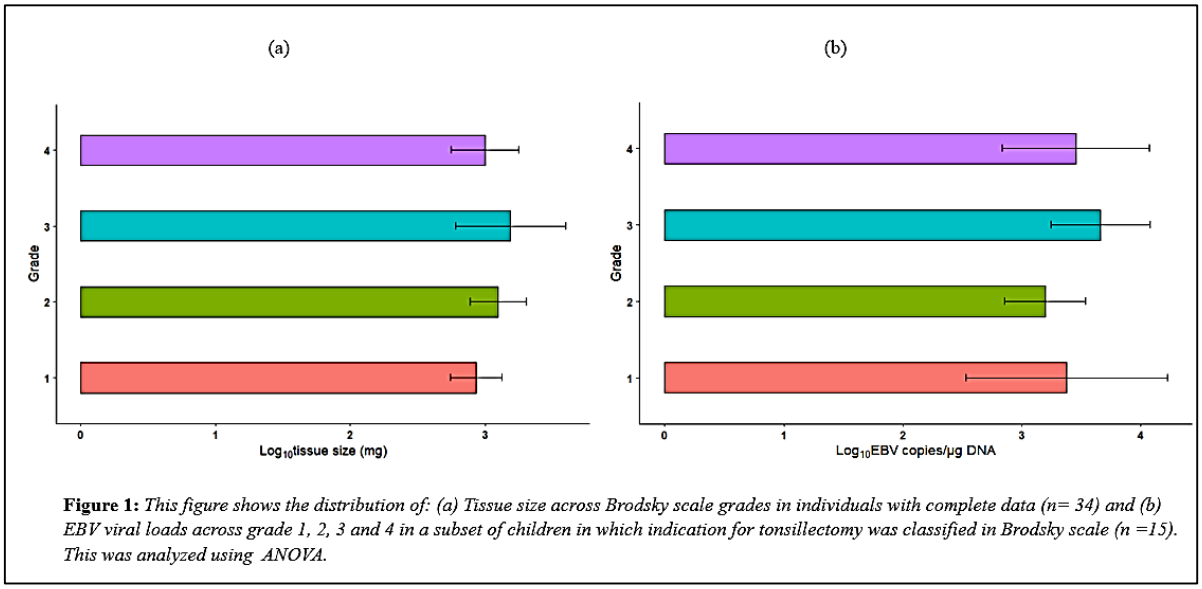

Figure 1: *This figure shows the distribution of: (a) Tissue size across Brodsky scale grades in individuals with complete data (n= 34) and (b) EBV viral loads across grade 1, 2, 3 and 4 in a subset of children in which indication for tonsillectomy was classified in Brodsky scale (n =15). This was analyzed using ANOVA.*

Most of the study participants underwent tonsillectomy due to adenotonsillar hypertrophy. Adenotonsillar hypertrophy, the enlargement of adenoids and tonsils, typically causes airway obstruction(37). This results in symptoms such as breathing difficulties, thus necessitating surgical intervention. Our observations are in agreement with earlier studies, which reported adenotonsillar hypertrophy as the main indication for tonsillectomy (37,38). However, findings differ from those of Han et al.'s study, which did not consider adenotonsillar hypertrophy as one of the indications for tonsillectomy (39).

In addition, we assessed the effect of EBV infection on clinical parameters by EBV status and observed lower haemoglobin levels among the EBV-positive compared to EBV-negative individuals, though still within normal range. This may relate to elevated hepcidin levels in EBV-positive children, which may affect iron metabolism (40). Hepcidin is a hormone that plays a critical role in the regulation of iron levels in the human body. It was earlier reported that iron chelation in EBV-positive epithelial cancers promoted viral replication (41). However, the role of EBV in iron regulation and its impact on haemoglobin remains unclear.

Furthermore, we investigated the impact of subclinical malaria infection on EBV detection. All participants in our study were negative for *P. falciparum* by RDT and blood smear at the time of recruitment. Unexpectedly, a higher proportion of children who tested negative for malaria were EBV positive, while only a small percentage of those who tested positive for malaria were EBV positive. Since all the children resided in a malaria holoendemic region, they have likely experienced multiple episodes of malaria throughout their lives. This repeated malaria infection could potentially impact their immune system and contribute to reactivation or persistence of EBV, resulting in higher viral loads. However, further investigation is needed to validate these results.

Existing research has shown that tissue size is a key factor in determining the need for surgical intervention in cases of tonsil enlargement, for both children and adults (42). As a result, getting a precise method for measuring tonsil size before the surgery procedure could aid in the selection of suitable candidates for treatment. Consistent with the literature, which indicates that the tonsils reduce in size as the children get older (43,44), we observed a significant positive association between tonsil tissue and age, suggesting that age may play a role in determining tonsil size. This relationship may further be clarified by the normal development and growth patterns of lymphoid organs, which increase during childhood as the immune system matures and is exposed to diseases.

We also found no significant association between tissue size and tonsil grading. Even though our results contrast with several earlier studies, which reported a positive correlation between tonsil grade and tonsil volume in adults and children (45–49), direct comparison was limited due to methodological differences; those studies measured volume via water displacement by tonsil tissue, whereas we assessed tissue weight in milligrams.

One limitation of this study is that we were not able to measure all nutritional elements, restricting our capacity to completely evaluate the role of diet in the observed health states and relationships.

Conclusion

In conclusion, a higher proportion of children who tested negative for malaria were EBV positive, suggesting a possible relationship between repeated malaria infections and EBV detection. Additionally, the low haemoglobin levels observed among EBV-positive children suggest that EBV infection may influence haemoglobin levels. However, this finding warrants further investigation.

Recommendations

We recommended in malaria holoendemic regions to better define the temporal relationships between EBV acquisition, persistence, viral load dynamics, and repeated malaria exposure.

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Author contribution

- EK: designed the study, collected samples, carried out the laboratory and data analysis, interpretation and drafted the manuscript.
- AA: designed the study and extensively revised the manuscript.
- IO: helped with data analysis and interpretation.
- KW: Collected samples and helped with the laboratory analysis and review of the manuscript.
- BO: Collected samples and helped with the laboratory analysis and review of the manuscript.
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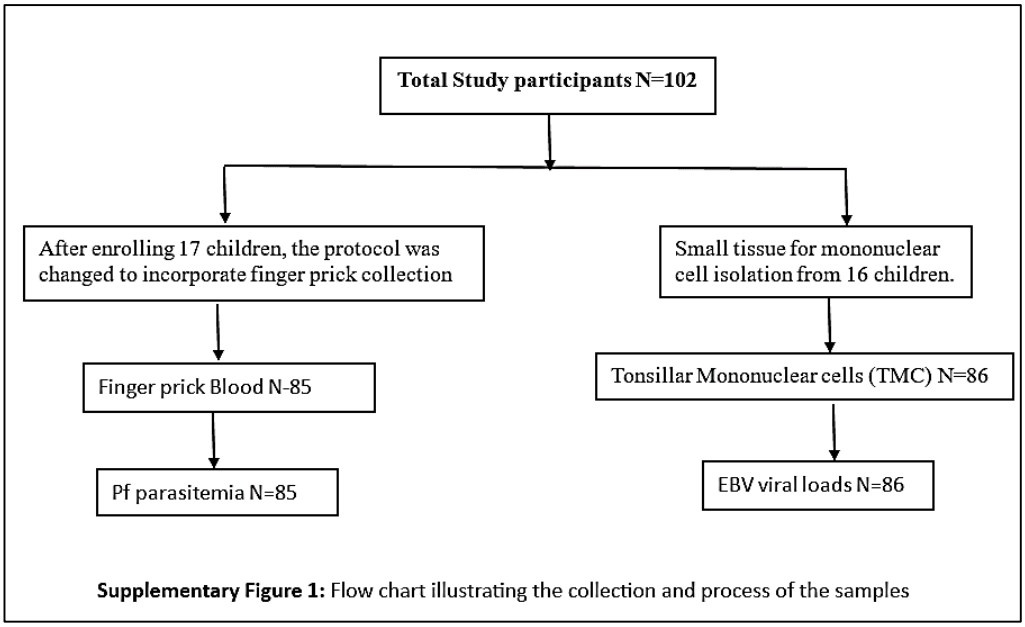
Supplementary Figures and Tables



Supplementary Figure 1: Flow chart illustrating the collection and process of the samples

Supplementary Table 1

Primer sequences

DNA Target	Sequences (5′-3′)
EBV Balf-5	CGG AAG CCC TCT GGA CTT C (Forward)
	CCC TGT TTA TCC GAT GGA ATG (Reverse)
β-Actin	TCA CCC ACA CTG TGC CCA TCT ACG A (Forward)
	CAG CGG AAC CGC TCA TTG CCA ATGG (Reverse)
EBNA-3C	AGA AGG GGA GCG TGT GTT G (Forward)
	GGC TCG TTT TTG ACG TCG G (Reverse)
Pf 18S	GTCAGCTCCGTGTCGATTG (Forward)
	TCCTCCATCTTTGTATTCTC (Reverse)