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Molecular Detection of Epstein Barr Virus Co-Infection in HIV Positive Women Attending Selected Hospitals in Sokoto, Nigeria

Raji, M.I.O. [*1], Balogun, D.O. [2], Riskuwa, M.L. [2], Ibrahim, A.D. [2] and Adisa-Raji, N.O. [1]
Department of Pharmaceutics and Pharmaceutical Microbiology, Faculty of Pharmaceutical Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria [1], Department of Microbiology, Faculty of Science, Usmanu Danfodiyo University, Sokoto, Nigeria [2]
Corresponding Author*: rajimuda@gmail.com, raji.mudasiru@udusok.edu.ng
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Abstract
Viral infections contribute a larger percentage of all human cancers. Epstein Barr virus (EBV) is a type of herpes virus with no clinical manifestation in majority of individuals. However, when it occurs in adulthood, it causes benign lymph proliferative disease known as mononucleosis. This study investigated the molecular detection of EBV co-infection among HIV positive women attending some selected hospitals in Sokoto, Nigeria. Questionnaires were administered on 80 consenting HIV positive women attending three selected hospitals in Sokoto to gather data on socio-demography and risk factors. Blood samples were aseptically collected by venipuncture from the HIV positive patients and EBV screening done using PCR analysis with primer +5′ ACAACCACTCATGATGCCAC (Forward), -5′ ACCGTGGTTCTGGACTATCT (Reverse) for Type 1 EBV (EBV-1) and primer +5′ GGTAGCCTTAGGACATACTC (Forward), -5′ TGGAGGGAGTCCTGTACTAT (Reverse) for Type 2 EBV (EBV-2). Result indicated that majority (63.75%) of the HIV positive women were in the age group of 21-30 years with majority not educated (56.25%) and largely (63.75%) from polygamous family. It further showed polygamy as a risk factor for co-infection of EBV in HIV positive women (P-value= 0.03). Four strains of EBV were detected from 40 out of 80 HIV positive blood samples that underwent molecular screening namely Multiple Strain EBV, Single Strain EBV, Type 1 EBV (EBV-1) and Type 1 & 2 EBV (EBV-1&2) with EBV-1 having the highest occurrences of 60.7%. Co-infection of EBV among HIV positive women attending the selected hospitals in Sokoto, Nigeria was established with EBV-1 having the highest prevalence and in subjects in polygamous relationships.

Keywords: Molecular detection, Epstein Barr virus co-infection, HIV positive women, Sokoto - Nigeria

Introduction

EBV is one of the most common viral infections in human. It is the etiological agent for infectious mononucleosis and is one of the most common viruses that are transmitted through the oral transfer of saliva (Smatti *et al.,* 2018). It is the most prolific viral contributor to the development of human lymphomas and is etiologically linked to multiple malignancies that includes nasopharyngeal carcinoma (NPC) in Southern Chinese people, a high incidence of Burkitt's lymphoma (BL) in sub-Saharan Africa and a high incidence of infectious mononucleosis in teenagers and young adults in western countries (Kimura *et al.*, 2013; Balfour *et al.*, 2015; Tsao *et al.*, 2015). Each of these exceptional geographical or demographic differences in disease incidence may be accounted for by other cofactors but there has long been interest in the possibility that genetic variation in the EBV in different parts of the world might play a role (Tzellos and Farrell, 2012). Nasopharyngeal cancer is a rare cancer worldwide. It is now found to be increasing in incidence in Nigeria, though the incidence is lower when compared to countries in the

Mediterranean basin, North Africa and Southeast Asia. This cancer is frequently associated with Epstein-Barr virus (Omoseebi *et al*., 2017). Two major types of EBV namely EBV-1 and EBV-2– have been identified and differ in geographic distribution. The role of specific EBV types in the etiology of different cancers is unknown. Immunocompromised patients more commonly harbour both subtypes of EBV (Abdullah and Al-Hamadany, 2014). EBV-2 maybe more common in Africa (Correia *et al.,* 2017). It is a contagious virus that is transmitted from person to person and occurs throughout the world (Jassim *et al.,* 2021).

EBV is produced in the saliva of persistently infected individuals, and transmission almost certainly occurs orally through close salivary contact during kissing, but the virus has also been reported in both male and female genital secretions, suggesting that sexual transmission may occur (Dunmire *et al.,* 2018). It may also be transmitted by blood transfusions or organ transplant. While for most people it poses no serious immediate consequences (other than misery from cold-like symptoms and fatigue), it can pose long-term health consequences. In a cohort study of sexually active young women, the development of detectable antibodies against EBV after primary infection increased with increasing number of sexual partners, and was greatest when a new sexual partner was encountered in the 2 years before seroconversion. In addition, transient EBV DNA loads were detected in cervical cytology samples in some of the women (Lam *et al*., 2018). Over the past decade several investigators have raised the possibility that Epstein Barr Virus may also be involved in the pathogenesis of breast carcinoma (BC), the most common carcinoma in females (Al-Fatlawi *et al.,* 2020). Multiple epidemiologic studies have identified Epstein-Bar virus (EBV)-directed antibodies in more than 90% of the human population (Dunmire *et al.,* 2018). However, virus isolation studies of certain T-cell immunocompromised, HIV-positive cohorts have shown that type 2 EBV exists in much greater proportion in these groups (Eliassen *et al.,* 2018). The predominance of a single transforming virus strain, most commonly type-1 EBV (EBV-1) rather than type-2 EBV (EBV-2), has been demonstrated in healthy individuals; however, increasing evidence suggests that multiple EBV infections are common within immunocompromised hosts (Neves *et al.,* 2017). In addition, type-1/type-2 EBV (EBV-1&2) co-infection has been demonstrated. The virus type can be determined at the DNA level by PCR amplification across these polymorphic loci (Correia *et al.,* 2018). Doctors rarely look at EBV as a cause of illness as patients are often asymptomatic of the virus as such, EBV isn't routinely checked (Worth *et al.,* 2016). Several risk factors which include solid organ and haematopoietic stem cell transplant recipients, living in tropical countries, having immune deficiency disorder like HIV and being sexually active have been associated with acquiring Epstein Barr Virus infection (White *et al.,* 2019). Laboratory diagnosis for detecting EBV is not routinely done in laboratories in Hospitals within Sokoto Metropolis; diagnostic kits are also not easily available and expensive. This research is therefore aimed at determining the preponderance of EBV co-infection in the HIV positive women attending selected hospitals in Sokoto, Nigeria through identification of susceptible groups and risk factors for Epstein Barr Virus infection.

Materials and Methods

Study area

The area that formed the scope of this study is Sokoto State, which is part of the Sokoto basin, North-western Nigeria. It is bordered to the North by Niger Republic and to the South by Kebbi and Zamfara States of Nigeria. It represents the South-eastern sector of the lullemmenden basin; which is a large synclinal structure trending NE-SE and it extends to Niger Republic, Mali, and Benin Republic. Sokoto Basin compromises one tenth of the lullemmenden basin (Kogbe, 1979). Sokoto State lies approximately between latitude 11°33'42" N and 13°59'7" N and longitude 4°9'36" E and 6°45'33" E.

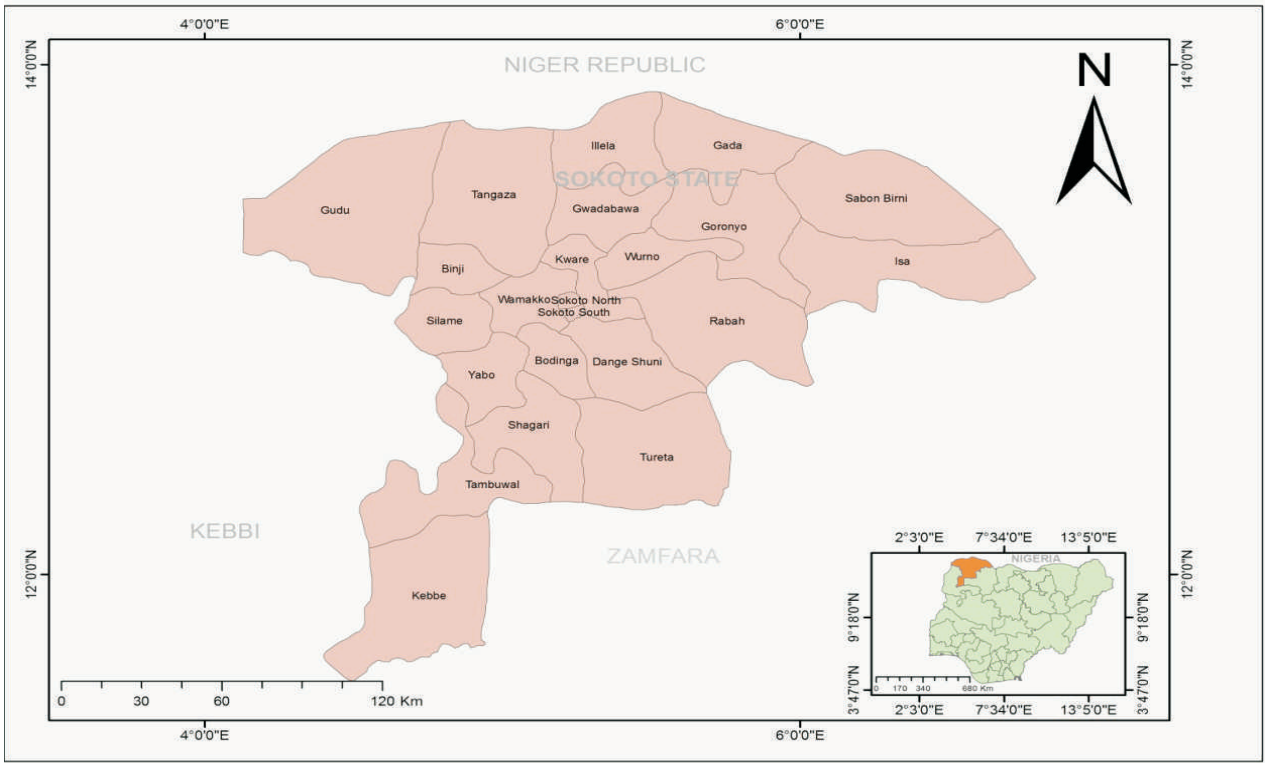

Figure 1: **Location of Sokoto State in map of Nigeria (insert) and Location map of Sokoto**
Source: Kogbe, C.A. (1979). Geology of Southeastern (Sokoto) sector.

Sample Area

The areas designated "H" meaning hospitals formed the sampling areas. Blood samples were collected from these three hospitals.

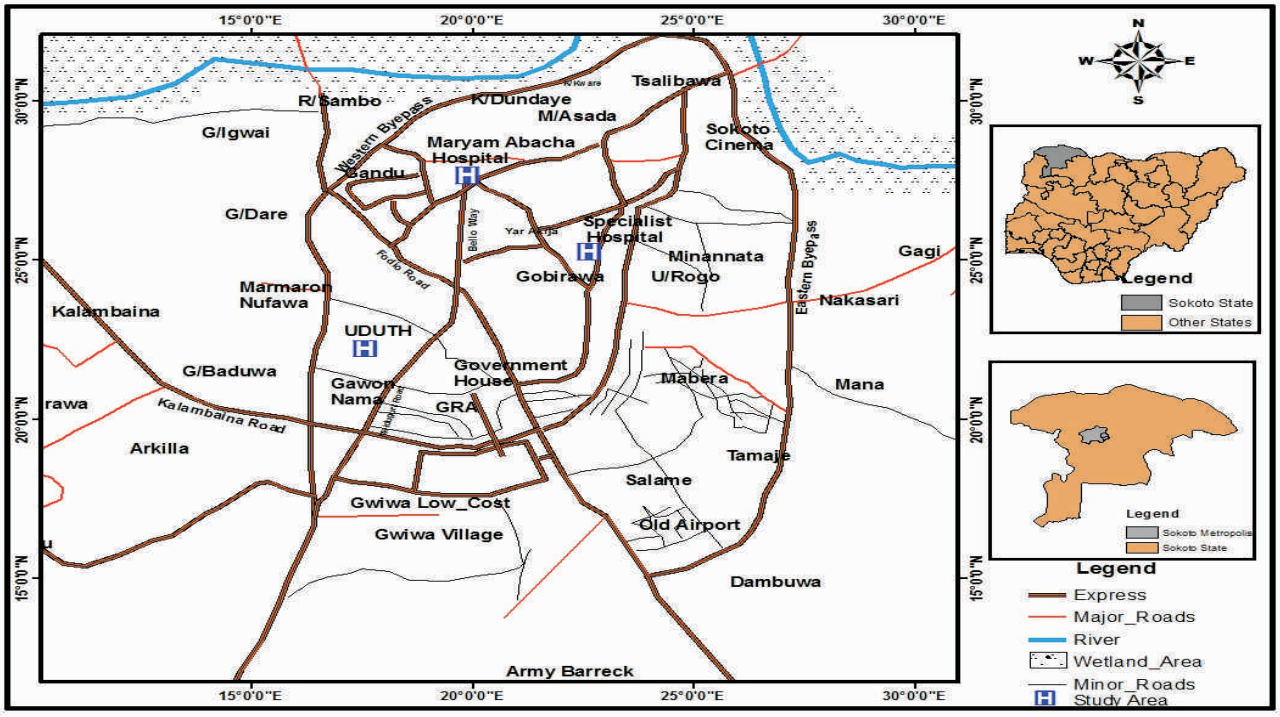

Figure 2: **Sample area of selected Hospitals in Sokoto**
Source: GIS Lab. Department of Geography UDUS 2018

Study population
The study population consisted of HIV positive women of different ages attending the following Hospitals within Sokoto Metropolis: Usmanu Danfodiyo University Teaching Hospital (UDUTH), Specialist Hospital and Maryam Abacha Women and Children Hospital. All those who declined to give consent for inclusion were excluded from the study.

Study design
The work is designed to comprise two parts – the first part bothers on the administration of questionnaire to gather background information regarding EBV co-infection in HIV positive women attending three selected hospitals in Sokoto, Nigeria; and the second part is on molecular analysis of the isolated EBV.

Questionnaire administration
A self-designed structured questionnaire was used to obtain clinical information and risk factors associated with Epstein Barr Virus. This was achieved through collaboration with experienced Laboratory Scientist personnel in the selected hospitals who served as research assistants. The questionnaires containing demographic information and possible risk factors were administered to all participants enrolled in the study.

Molecular analysis
Sample size determination
Prevalence of Epstein Barr Virus (EBV) in HIV positive women attending the selected hospitals within Sokoto Metropolis is unknown. This research was conducted using 80 samples of HIV positive women from three selected hospitals within Sokoto metropolis based on availability and accessibility within the period of three months (April to July, 2021) according to the sampling method of Elfil and Negida (2017) in clinical research.

Sample collection
Ethical approval was sought for and obtained from the Usmanu Danfodiyo University Sokoto, Specialist Hospital Sokoto and Maryam Abacha Women and Children Hospital. At the respective hospitals, arrangement was made with the clinicians whereby subjects who consent to the study were selected. From each selected subject, two (2) millilitres of venous blood sample were collected by the research assistant using a sterile disposable syringe and needle under sterile conditions. The blood samples were emptied into an EDTA bottle, appropriately labelled and transported immediately to the Center for Advanced Medical Research and Training (CAMRET) Laboratory, Usmanu Danfodiyo University Teaching Hospital, Sokoto and stored in a Haier Thermocool Deep freezer (Model HTF-319H) at -20°C until required for further analysis (Patel *et al.,* 2021).

DNA extraction
Quick-DNA™ Miniprep plus kit was used to extract DNA from the blood samples collected according to the manufacturer's instructions. Exactly one (1) millilitre (ml) of blood samples collected from each patient was added to 1000µl of lysis buffer. Each of the selected sample preparations were mixed thoroughly using a vortex mixer (Dea XHIB Vortex Maxi mix II Thermolyne, UK) to obtain a uniform suspension. The samples were then incubated for 10 minutes at 56ºC using Block heater (Stuart Scientific SB160, Bibby Sterilin Ltd CAT No SB 160, UK). The samples were then transferred to a Zymo-Spin Column in a collection tube. The columns were centrifuged for 1 min at 10000×g (Eppendorf Centrifuge 5417R, UK) high speed micro centrifuge. The collection tube containing the flow-through solution was discarded, the Zymo-Spin Column was then placed into new collection tubes. Then, 200µl of pre-wash buffer was added to each of the collection tubes and centrifuged for 1min at 10000 × g. The flow-through was discarded and the purification column placed back into the collection tube. Genomic DNA (500µl) wash buffer was added to each of the column and centrifuged for 1min at 10000×g. The collection tubes containing the flow-through solution was discarded and the Zymo-Spin Column was transferred to a sterile 1.5 millilitres (ml) micro centrifuge tube. Then, 100µl of elution buffer was added to the center of each of the Zymo-Spin Column membrane to elute genomic DNA. The preparation was incubated for 5 minutes (mins) at room temperature (27 °C) and then centrifuged at top speed (≤ 12000 ×g) for 30 seconds (secs). The

purification column was discarded; the purity and quality of the DNA was ascertained using BioSpec nano-drop spectrophotometer (Shimadzu Biotech Spectrophotometer A116450, Japan) then, placed in aliquot of 50µl in tubes and stored at -20ºC.

EBV screening using PCR

EBV screening was carried out by Polymerase Chain Reaction (PCR) using Kyratec Super cycler (Model SC-300, Australia) according to the method of Smatti *et al.* (2017). The polymerase chain reaction (PCR) was used to amplify a 110 base pair DNA fragment in Bam HIW highly conserved large internal repeat region within the EBV. For type l-specific PCR (EBV1), primers +5' ACAACCACTCATGATGCCAC (2079-2098) and -5'ACCGTGGTTCTGGACTATCT (2338-2319) were used while primers +5'GGTAGCCTTAGGACATACTC (2088-2107) and -5' TGGAGGGAGTCCTGTACTAT (2340-2321) were used for type 2-specific PCR (EBV2). PCR amplification was performed in 50µL total reaction volume containing 300 ng of DNA or 50ng of control, 1µL of each primer, 0.2mM of each dNTPs, 0.5-unit thermostable Taq DNA polymerase and 1 x Reaction buffer (all reagents were supplied by Promega, USA). DNA positive control for EBV was derived from patients' samples known to harbour the virus and nuclease free distilled water replacing DNA was used as negative control. Cycling conditions were performed as follows: one initial denaturation step at 94ºC for 3 minutes; 30 cycles of denaturation at 92ºC for 30 seconds, annealing at 45ºC for 1 minute and extension at 72ºC for 1 minute; and one final extension step at 72ºC for 10 minutes.

Agarose gel electrophoresis

The agarose gel electrophoresis was performed according to Green and Sambrook (2019). Aliqout (100ml) of Tris-Borate EDTA buffer was added to 900ml of nuclease free distilled water to make 1 x Tris-BE (TBE) buffer. Then, 1.5g of agarose was dissolved in 100ml of 1 x Tris buffer and microwaved for 30 - 60s to melt the agarose. Thereafter 5µl of ethidium bromide was added and mixed thoroughly to obtain a homogenous mixture. The mixture was then poured into the gel caster and the comb inserted and allowed to solidify. PCR products (5µl) were electrophoresed in 1.5% agarose gel. DNA marker 100bp (Fermenter) was used as the molecular size marker. The gel was run at 90V for 1 hour and the amplicons were visualized under automatic molecular imaging trans-illuminator (Gel DOC™ XR+ BIORAD, U.S.A) to check for the presence of the EBV PCR products of EBV-1 and EBV-2 at 120bp and 860bp respectively and the image was stored in the gel documentation system.

Results

Questionnaire administration

Characteristic pattern of study participants

Eighty (80) patients enrolled and tested to ascertain the incidence of EBV infection among HIV infected women in three selected hospitals within Sokoto Metropolis. Majority of the study participants 51(63.75%) belonged to 21-30 years of age, followed by 31-40 years of age with 22(27.5%), and age 40 and above of the study participants were 7 (8.75%). Large number (56.25%) of the study participants had no educational background, 30% had primary education, 10% had secondary education and 3.75% had tertiary education. The result of Type of Marriage showed that out of the 83.75% married participants, 63.75% of the participants were practicing polygamy while 20% of the participants were practicing monogamy; however, 16.25% of the participants were not married (Table 1).

Table 1: Characteristic Pattern of HIV Positive Women attending selected Hospitals in Sokoto

Characteristic	Parameter	Frequency (%)
Age (Years)	21 – 30	51 (63.75)
	31 – 40	22 (27.5)
	41 and above	7 (8.75)
	Total	80 (100)
Education	Primary Education	24 (30)
	Secondary Education	8 (10)
	Tertiary Education	3 (3.75)
	None Education	45 (56.25)
	Total	80 (100)
Type of Marriage	Monogamy	16 (20)
	Polygamy	51 (63.75)
	Not married	13 (16.25)
	Total	80 (100)

The results of associated risk factors among the study participants attending UDUTH, Specialist Hospital and Maryam Abacha Women and Children Hospital within Sokoto Metropolis is presented in Table 2. Out of 16 participants practising monogamy type of marriage, 9 were EBV positive and 7 were EBV negative, while for polygamy type of marriage, 29 were EBV positive and 22 were EBV negative out of a total of 51 participants. All the 9 divorced participants were EBV negative while 2 out of 3 re-married participants were EBV negative with 1 being EBV positive. All 11 participants that had taken blood transfusion were EBV negative while all 7 participants that had gone through surgical operation were also EBV negative. However, 2 out of 3 participating pregnant women were EBV negative while 1 was positive for EBV.

Table 2: Associated Risk Factors among HIV Positive Women attending selected Hospitals in Sokoto

Risk Factors	No of Participants	EBV Positive (%)	EBV Negative (%)	P-Value
Monogamy	16	9 (56)	7 (44)	0.08
Polygamy	51	29 (57)	22 (43)	0.03
Divorced	9	0 (0)	9 (100)	0.50
Re-married	3	1 (33)	2 (67)	0.30
Blood transfusion	11	0 (0)	11 (100)	0.50
Surgical operation	7	0 (0)	7 (100)	0.50
Pregnant	3	1 (33)	2 (67)	0.30
Total	80	40	40	

P-value = < 0.05

Molecular analysis on prevalence of EBV in HIV positive women

The result of molecular analysis as contained in Table 3 shows that 40(50%) of the 80 HIV positive blood samples were EBV positive with highest prevalence of 60.7% in EBV-1. Multiple strain EBV had 46.9%, Single strain EBV had 33.3% while EBV-1&2 had 20%.

Table 3: Prevalence of EBV in HIV positive women attending selected Hospitals in Sokoto

	EBV DNA Status			Percentage	
EBV Type	Positive	Negative	Total Sample	% Positive	% Negative
Multiple strain EBV	15	17	32	46.9	53.1
Single strai n EBV	6	12	18	33.3	66.7
EBV-1	17	11	28	60.7	29.3
EBV-1&2	2	0	2	20	0
Total	40	40	80	50	50

Molecular analysis showing EBV Types

Figures 1 – 4 show the EBV types detected in HIV positive women in the selected hospitals in Sokoto. All experiments included DNA from HIV positive blood samples as a positive control (Samples) a no template control (NT) and M the molecular weight marker. In Figure 1, the result showed that 15 out of 32 patients demonstrated infection with multiple strains (dimers and trimmers) of EBV (samples 1, 5, 7, 10, 11, 16, 17, 18, 19, 20, 21, 23, 24, 25 and 26) while 6 out of 18 samples were positive for the same strain in Figure 2. In Figure 3, samples 51 - 57, 59 and 60 detected the presence of EBV-1 with PCR product at about 120 bp while EBV-2 were detected in samples 61 - 70. EBV strains were seen in all the samples in Figure 4, however, EBV2 were detected only in two samples (78 and 80).

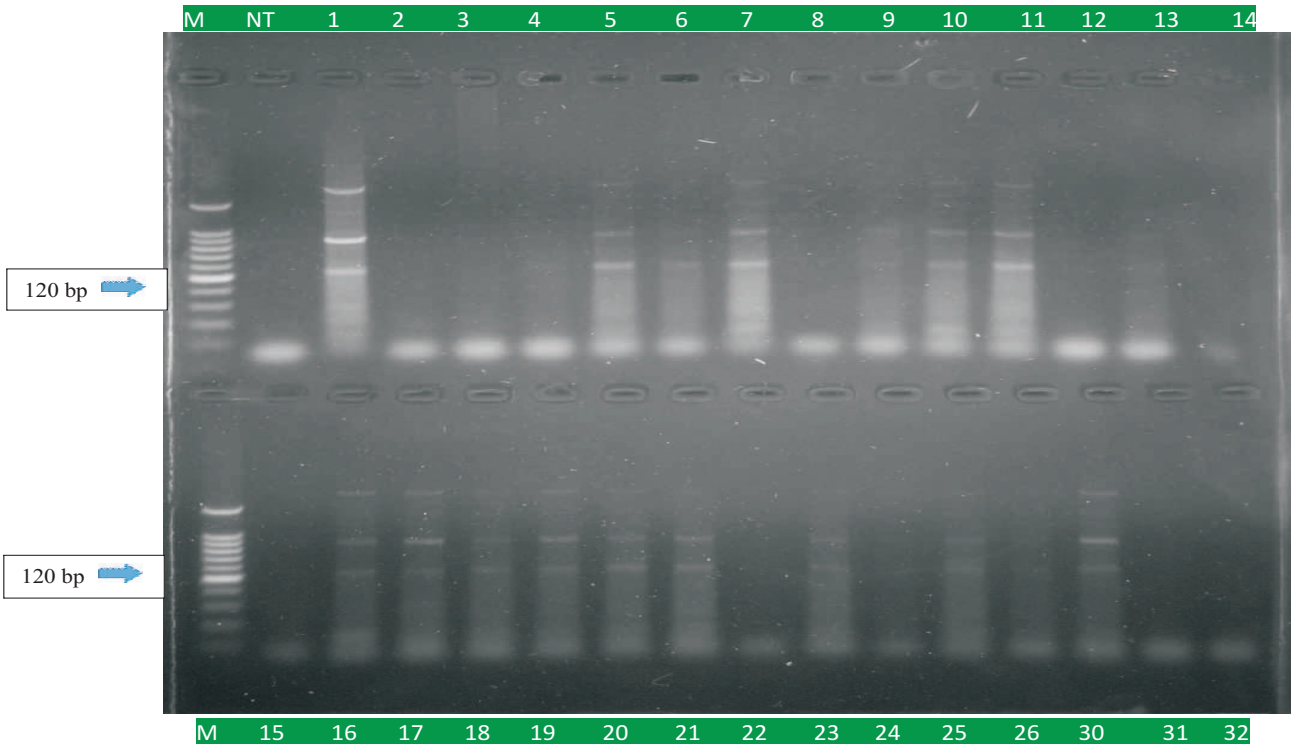

Figure 1: Multiplex PCR detection of EBV in HIV positive women attending selected hospitals in Sokoto

Row 1: Well 1= Marker, Well 2 = No template control, Well 3 = Sample 1 to 14
Row 2: Well 1= Marker, Well 15 to 32 = Samples
M = 100 bp DNA Ladder

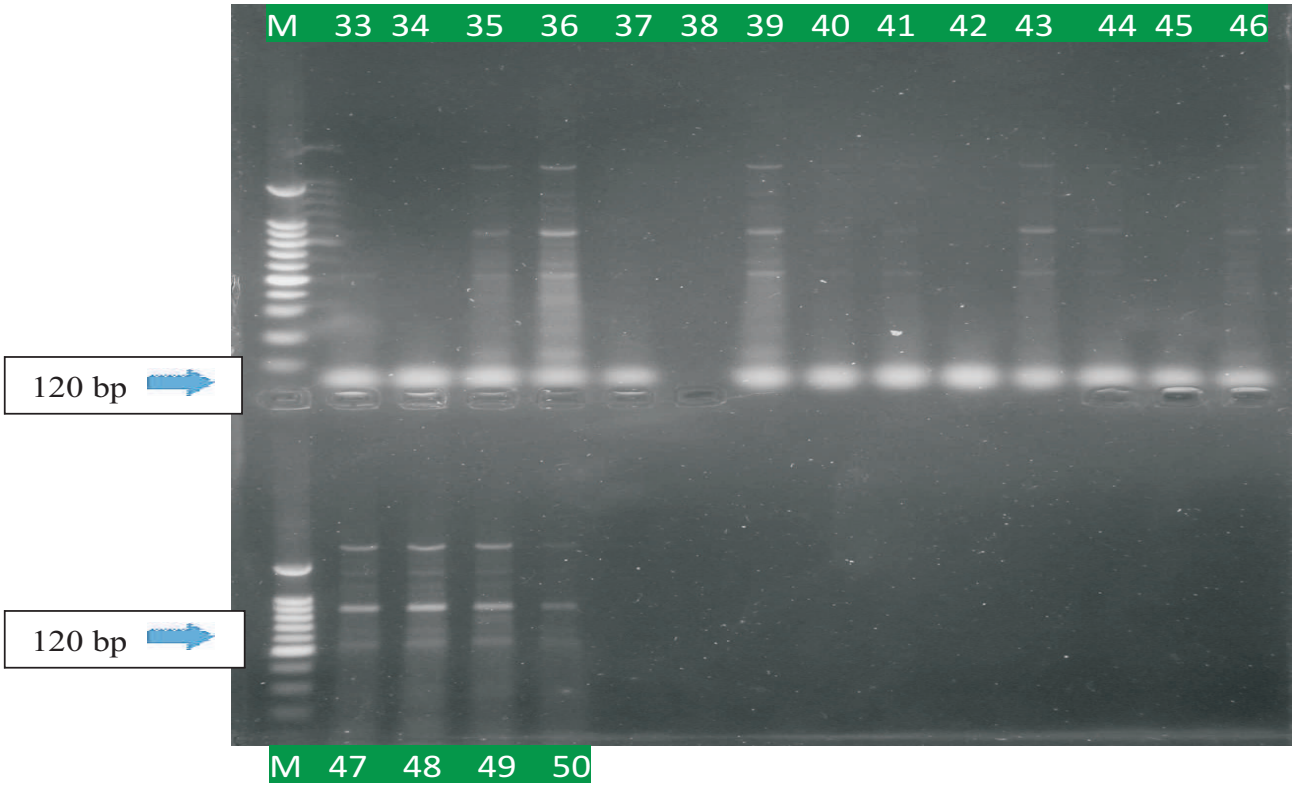

Figure 2: Multiplex PCR detection of EBV in HIV positive women attending selected hospitals in Sokoto

Row 1: Well M = Marker, Well 33-37 = Samples, Well 38 = Blank, Well 39 – 46 = Samples
Row 2: Well M = Marker, Well 47-50 = Samples

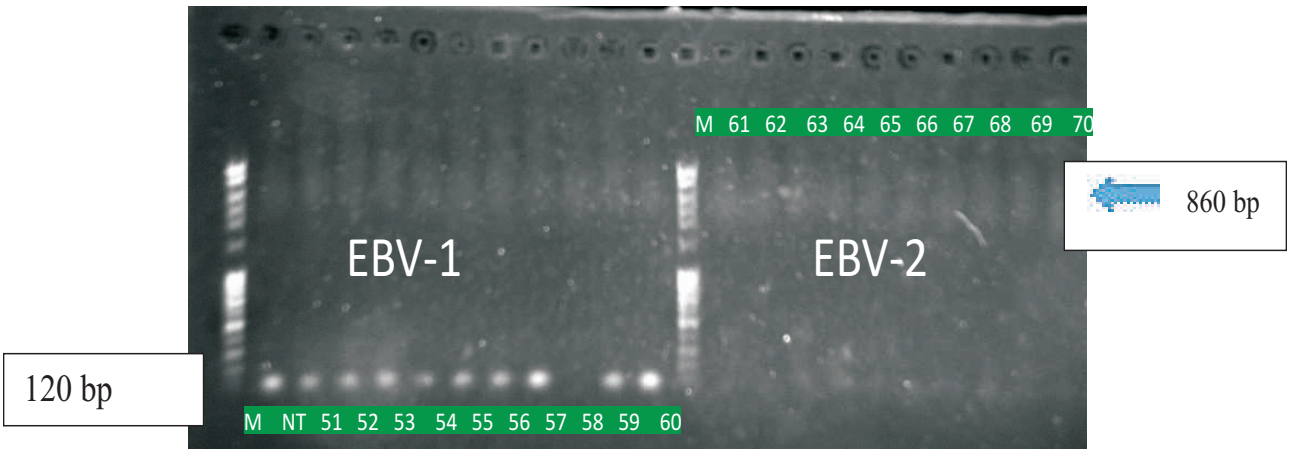

Figure 3: Singleplex PCR detection of EBV-1 and EBV-2 in HIV positive women attending selected hospitals in Sokoto

M = 100 bp DNA Ladder, NT = no template control
EBV1 (120 bp amplicon) seen in samples (51 - 60), Lane 58 is empty
EBV2 (860 bp amplicon) seen in samples (61 - 70)

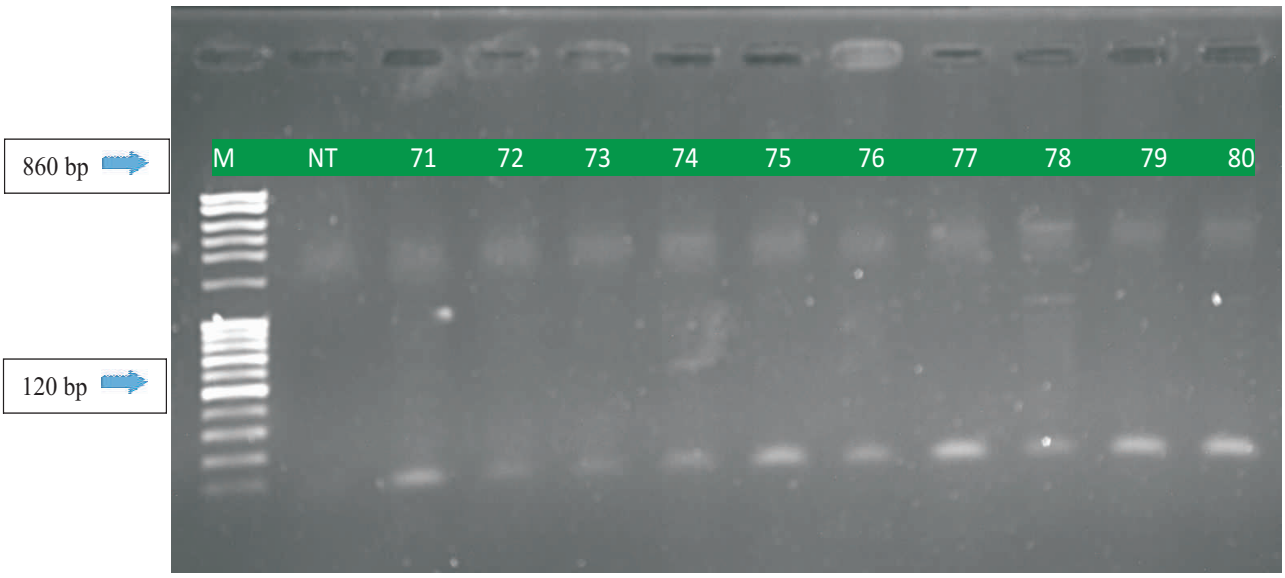

Figure 4: Multiplex PCR detection of EBV-1 and EBV-2 in HIV positive women attending selected hospitals in Sokoto

M = 100 bp DNA Ladder
NT = no template control
EBV-1 (120 bp amplicon) seen in all the samples and EBV-2 (860 bp amplicon) seen in samples 78 and 80
Lanes 71 - 77 and 79 positive results for EBV-1. Lanes 78 and 80 positive results for EBV-2

Discussion

Epstein Barr virus (EBV) is one of the leading causes of death and opportunistic infection that play an important role in HIV-positive individuals due to immunosuppression. The appearance of this virus in HIV positive individuals according to Paez-Nova *et al.* (2021), indicates the deterioration of HIV infection.

In this study, 80 samples from HIV infected individuals were tested and the result indicated that age had an influence on the prevalence of infection. The outcome of the result can be attributed to geographical location, sexual activity as well as low living standard of the study subjects. Considering the gender prevalence, the highest prevalence of EBV was found among the females in accordance with the findings of Hjalgrim *et al*. (2007b). It might be as a result of high record of sexual activeness in females than their male counterpart in the study area. The overall prevalence of Epstein-Bar Virus among women living with HIV is 40(50%) out of the 80 samples collected in the period of the research as indicated in Table 3.

Polygamy is said to be an important determinant of EBV infection (Hjalgrim *et al*., 2007b). A larger number, 29 out of 51 of the women from polygamous family were positive for Epstein-Bar virus. This result therefore accounts for high level EBV infections attributable to polygamous type of marriage among the study subjects. The high rate of EBV in the women from polygamous family may be as a result of high risk of exposure from other co-wives through the husband as the husband might serve as a means of infecting women who are co-wives to an already infected woman. Nevertheless, an overcrowded polygamous family setting may be a risk factor without necessarily considering the sexual life of the family. This crowding nature may also be accompanied with pre-chewing food practices for infants as a factor for the acquisition of the EBV virus at tender age. Therefore, transmission in the polygamous family may be as a result of intimate oral exposure as saliva harbours the virus. This agrees with Hjalgrim *et al.* (2007a) who described the infection as something that can be delayed to adolescence.

It is clear from previous literature that HIV is a

factor for opportunistic diseases to set in after the immune system must have depleted and women who were the subjects of the current study were HIV positive group hence, an increased chance to the prevalence of Epstein bar virus. Participants in this study within ages 21-30 had a higher percentage (63.75%) of HIV positive status. This result is in accordance with the distribution of HIV infection in the general population, where the prevalence of HIV is higher in the higher age-groups, according to Anoubissi *et al.* (2019) where it is stated that women aged 21-30 are at least two times more at risk of being infected with HIV compared with those that are older. The high prevalence of EBV in this study with 50% of the 80 total samples may be as a result of their sexually active age of 21-30 and 31- 40 when compared to those who are above the age of 40. This result is also in agreement to that of Mandal et *al*. (2021) who observed in their work that HIV is a risk factor to EBV and are more common among sexually active age. It is therefore important for HIV and AIDS prevention programmes targeted at young persons aged 21 and above to include promoting the delay of sexual debut as this predisposes them, especially young females, to multiple sexual partnerships, which in turn puts them at risk of HIV infection. It is also important to design bold and effective interventions that address the needs of all young persons aged 15 and above in Sokoto as well as Nigeria and in other sub-Saharan countries such as South Africa, Botswana and Malawi, which are still characterized by a high burden of HIV-induced morbidity and mortality.

The improvement in health care delivery is shown in this study because transfusion of blood which was previously known as a common factor of infection had no contributing factor to EBV transmission in this study. Among the 3 pregnant women tested, there was 1 positive result for EBV. The study recorded no positive result for EBV from blood transfusion, surgical operation, and divorced state. This is therefore in agreement with the study conducted by Styczynski *et al. (*2013) who found in their work that blood transfusion and surgical operation are not risk factors in the EBV infection. More recently, Teijido *et al.* (2020) also described it as an infection with rare risk from blood transfusion. Although, it disagrees with the work of Babel *et al. (*2005) who claimed that blood transfusion and surgical operation are common factor to EBV infection transmission.

In this study, the incidence rate among the study showed a decrease in the number of positive EBV in relation to academic qualification as low number of women were positive among those that attended higher education of learning. This may perhaps be because of exposure and use of information about the etiquettes of interaction in a large population.

The result of PCR analysis showing EBV-1 as the most prevalent (60.70%) in this study is in agreement with that of Leonn *et al*. (2022) with prevalence value of 47.37% and that of Mandal *et al*. (2021) with value of 37.00%. There is therefore the need for treating EBV infection alongside the treatment of HIV for a better clinical outcome.

This study bears some limitation such as small sample size which may not permit generalization of our findings. Further larger study is needed to investigate both prevalence and incidence among the general and target populations.

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