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HIV-1 p24 antigen, HIV-1 protease and expression of HIV-1 Gag-Pol genes amongst HIV-1 Infected individuals in Federal Medical Centre, Lokoja, Nigeria.

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Abstract

This study is aimed at expressing HIV-1 Pol and Gag genes in the monitoring of HIV-1 patients on HAART in Federal Medical Centre, Lokoja, Kogi State, Nigeria, that randomly recruited 75 participants within the age range of 18 and 65 years with a population- based sample of males and females divided into three groups designated A to C; comprising of 25 HIV-1 seropositive individuals on HAART (TDF + 3TC + EFV) designated as group A, 25 HIV-1 seropositive individuals on HAART (AZT + 3TC +NVP) designated as group B, and 25 HAART naïve HIV-1 seropositive individuals designated as control group C. Blood samples were taken and analyzed using ELISA for p24 antigen, HIV-1 protease and reverse transcription-polymerase chain reaction for HIV-1 Gag-Pol genes. The PCR products were analyzed on 1% agarose gel electrophoresis. Data were analyzed using SPSS software application (version 25.0) and expressed as mean ± standard error of mean. The results showed a significant difference in p24 antigen and HIV-1 protease in group A (9.64 ± 5.34 and 5.48 ± 6.62) when compared to control (16.3 ± 3.68 and 26.68 ± 11.71) ($p<0.05$). There was also a significant difference in p24 antigen and HIV-1 protease in group B (13.01 ± 5.19 and 20.47 ±7.53) when compared to control (16.3 ± 3.68 and 26.68 ± 11.71) ($p<0.05$). The gene expression of HIV-1 Pol is broadly expressed compared to the HIV-1 Gag gene expression. The negative Gag expression could be due to decrease in p24 antigen as the antibody increases.

Keywords: HIV-1 POL; HIV-1 GAG; HIV-1 P24; HIV-1 Protease; HAART

1. Introduction

Human immunodeficiency virus infection has the ability to progressively shut down the host immune system due to exponential growth of the virus in some infected individuals [21]. This may result in the manifestation of acquired immune deficiency syndrome (AIDS) if HIV progression in a host is not put under control [21].

The HIV/AIDS pandemic has severely affected health development and eroded improvements in life expectancy, particularly in developing countries with the highest prevalence of infection [32]. Expanding access to antiretroviral therapy in resource-limited settings along with close monitoring is needed for successful treatment outcomes [1]. In high income settings, this is achieved by performing quantitative viral load monitoring every 3-6months [1]. However,

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in resource limited settings, patient evaluation is based on $CD4^+$ T-cell count, clinical findings, neither of which accurately predicts viral suppression [17].

In the past, before the availability of viral load testing, the P24 antigen assay was used extensively for monitoring the development of AIDS and for charting disease progression. The use of p24 antigen in predicting who may rapidly progress to AIDS (rapid progression) by noting a higher prevalence of P24 antigenemia at the first seropositive visit has been reported [8]. Earlier, it was reported that a decline in the serum titer of antibody to the p24 protein was suggestive of good prognosis but a rise in p24 antigen in HIV-positive individuals was an indication of progression to the most serious phase of the disease [5].

HIV-1 protease can serve as an alternative to HIV-1-RNA viral load because HIV-1 protease can be assayed in an ELISA based format; this is possible in resource limited settings [10]. In the management of HIV-1 infected individuals; p24 antigen and protease enzyme activities are not commonly used for monitoring treatment success or disease progression, therefore, developing alternative biomarkers such as p24 antigen, HIV-1 protease with their gene expressions may indicate treatment failure or success in our clinical settings which could help in the proper prognosis and management of HIV-1 infected patients [17].

2. Materials and Methods

2.1. Study Site

The study was carried out on HIV-1 infected individuals at Federal Medical Centre, Lokoja, Kogi State, Nigeria.

2.2. Study Design

The study was a cross sectional study that randomly recruited 75 individuals at the HAART Clinic of Federal Medical Centre Lokoja, Kogi State, Nigeria. A written informed consent was obtained from the subjects after approval by the ethics committee of the Kogi State Ministry of Health, Kogi State, Lokoja, Nigeria. Sample was taken from each participant at the start of research and each participant was known to be on antiretroviral therapy for at least two years except control subjects who were yet to commence drugs. Sample were analyzed for the following parameters; HIV-1 P24 antigen, HIV-1 protease, HIV-1 RNA Pol and Gag expression using agarose gel electrophoresis.

2.3. Inclusion and exclusion criteria

The study included both male and female confirmed HIV seropositive test participants without treatment failure placed on HAART for at least one year and newly diagnosed HAART naive male and female participants as control group. Individuals with known treatment failure and are above 65 years and below 18 years of age were excluded from the study. Individuals with comorbidities such as diabetes, cardiovascular disease and tuberculosis were excluded from the study.

2.4. Sample Collection

Five milliliters (5mls) of venous blood was collected from each subject for the research. Four milliliters (4mls) of blood was dispensed into Lithium heparin bottle for HIV-1 p24 antigen and HIV-1 protease while 1ml of whole blood sample was dispensed into 1ml of RNA shield for HIV-1 Gag and POL genes expression. Sample when taken, were centrifuged immediately at 3000rpm for 5 minutes, plasma obtained were stored at -20^0C before analysis.

2.5. Methods

The HIV status of the subjects were confirmed using Geenius HIV confirmatory system. The diagnosis of HIV and the criteria for commencement of HAART was based on serial algorithm which is Nigeria National guidelines for adult HIV and AIDS treatment and care [9].

2.5.1. Determination of p24 Antigen Using Enzyme Linked Immunoassay described by [2, 3].

Assay Principle: An anti-HIV p24 monoclonal coating antibody is adsorbed onto a microtiter plate. p24 antigen present in the sample or standard binds to the antibodies adsorbed on the plate; a FITC-conjugated mouse anti-p24 antibody added and binds to p24 antigen captured by the first antibody. Following incubation and wash steps, a HRP –conjugated mouse anti-FITC antibody is added and binds to the FITC conjugated anti-p24. Following unbound HRP-conjugated mouse anti-FITC antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A colored product is formed in proportion to the amount of p24 antigen present in the sample. The reaction is

terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from recombinant HIV-1 p24 protein and sample p24 concentration is then determined.

2.5.2. Determination of HIV-1 protease enzyme by Fluorometry using fluorescence Microplate Reader described by [17]

Assay Principle:

HIV-Protease activity assay kit utilize the ability of active HIV-protease to cleave a synthetic peptide substrate to release the free fluorophore which can be easily quantified (Ex/Em=330/450nm) using a fluorometer or fluorescence microplate reader.

FI – Substrate $\xrightarrow{\text{HIV-1 Protease}}$ Cleaved substrate + FI (Fluorescence) (Ex/Em = 330/450nm)

2.5.3. HIV-1 POL – GAG Gene Expression

Principle:

Principle of Polymerase Chain Reaction with HIV-1 RNA Targets [18]

The principle is based on the steps of RT – PCR (Reverse Transcription - PCR).

2.5.4. Detection of expressed genes

- At the end of the PCR cycles, the amplicons are denatured and are subsequently detected.
- Detected by Agarose Gel Electrophoresis.
- The targeted genes were visualized by Wealtec Dolphin-Doc UV transilluminator and photographed.

Reagents and Materials

Product code	Product description
ZR R1100 – 50	DNA/RNA shield (50ml)
IB OL0001	2 Olignucleotide oiolymole scale
NEB E5315S	One Taq One Step RT-PCR kit – 30 reactions
ZR R1057	Quik – RNA™ Miniprep plus kit (10 preps)

Specification of primers (Oligonucleotides)

Name	Ref No	Sequence	Bases
POL2316F	IBOL0001	GCTCTATTAGATACAGGAGCAG	22
POL2316R	IBOL0001	GCCTTGCCAGCACGCTCAGGC	21
HIV-1 GAG-F	1043371	AGTAAGAATGTATAGCCCTACCAGCAT	27
HIV-1 GAG-R	1043371	CTTAGAGTTTTATAGAACCGGTCTACATAGTC	32

GEL Loading Dye (4ml)

Quick –load purple DNA ladder

Polymerase Chain Reaction Methods [18].

Total RNA was extracted using Zymo-RESEARCH (ZR) whole – blood – RNA Miniprep with catalog number R1057 and R1058 by Zymo-RESEARCH CORPORATION according to manufacturer's specification at Iykenson Medical and Diagnostic Laboratory, Nnamdi Azikiwe University temporary site, Anambra State, Nigeria. The total RNA extracted was used immediately after extraction.

Total RNA Extraction for HIV-1 Using ZYMO-RESEARCH Whole-Blood RNA Miniprep [18].

Reverse Transcriptase and Amplification for HIV-1 POL:

- One Taq One-Step Reverse Transcriptase
- Polymerase Chain Reaction;

The extracted total RNA was retro-transcribed and amplified using One Taq One Step RT-PCR kit with catalog number NEB E5315S by Inqaba Biotec West Africa Limited according to the manufacturer's specification. HIV-1 POL genes (POL 2316F and POL 2316R) forwarded and reverse primers $\left(\frac{\text{GCTCTATTAGATACAGGAGCAG}}{\text{GCCTTGCCAGCACGCTCAGGC}}\right)$ were used to target HIV-1 POL template using Applied Biosystems 2720 thermal Cycler polymerase chain reaction machine at the Iykenson Medicals and Diagnostics Laboratory, Nnamdi Azikiwe University temporary site, Awka, Anambra State, Nigeria.

The system components were thaw and mixed by inverting ten times. The PCR was performed in a 50µl reaction mixture containing 25µl One Taq One-Step reaction master mix (2x), 2µl one Taq One-Step enzyme mix (2x), 2µl of each gene-specific forward primer (10µM), 2µl of each gene-specific reverse primer (10µM), 9µl of nuclease – free water and 10µl of the RNA template(s) was added last. The PCR was started immediately as follows: Reverse transcriptase at 48^0C for 30 minutes, initial denaturation at 94^0C for 1 minute, denaturation at 94^0C for 15 seconds, annealing at 54^0C for 30 seconds, extension at 68^0C for 1 minutes, Go to the denaturation step for 39 cycles, final extension at 68^0C for 5 minutes and final holding at 4^0C forever. 5µl five microlitre of the amplified PCR products were analyzed on 1% agarose gel containing ethidium bromide in IX Tris EDTA buffer. Electrophoresis was performed at 90 volt for 30 minutes with the EDVOTEK tetra source electrophoresis machine, Bethesda, USA. The targeted genes (HIV-1 POL) were visualized by Wealtec Dolphin – DOC UV transilluminator and photographed. The molecular weights were calculated using molecular weight standard of the marker.

Reverse transcription and amplification for HIV-1 GAG

- One Taq One-Step Reverse Transcriptase
- Polymerase Chain Reaction;

The extracted total RNA was retro-transcribed and amplified using One Taq One Step RT-PCR kit with catalog number NEB E5315S by Inqaba Biotec West Africa Limited according to the manufacturer's specification. HIV-1GAG genes (HIV-1 GAG-F and HIV-1GAG-R) forwarded and reverse primers $\left(\frac{\text{AGTAAGAATGTATAGCCCTACCAGCAT}}{\text{CTTAGAGTTTTATAGAACCGGTCTACATAGTC}}\right)$ were used to target HIV-1 GAG template using Applied Biosystems 2720 thermal Cycler polymerase chain reaction machine at the Iykenson Medicals and Diagnostics Laboratory, Nnamdi Azikiwe University temporary site, Awka, Anambra State, Nigeria.

The system components were thaw and mixed by inverting ten times. The PCR was performed in a 50µl reaction mixture containing 25µl One Taq One-Step reaction master mix (2x), 2µl one Taq One-Step enzyme mix (2x), 2µl of each gene-specific forward primer (10µM), 2µl of each gene-specific reverse primer (10µM), 9µl of nuclease – free water and 10µl of the RNA template(s) was added last. The PCR was started immediately as follows: Reverse transcriptase at 48^0C for 30 minutes, initial denaturation at 94^0C for 1 minute, denaturation at 94^0C for 15 seconds, annealing at 54^0C for 30 seconds, extension at 68^0C for 1 minutes, Go to the denaturation step for 39 cycles, final extension at 68^0C for 5 minutes and final holding at 4^0C forever. 5µl five microlitre of the amplified PCR products were analyzed on 1% agarose gel containing ethidium bromide in IX Tris EDTA buffer. Electrophoresis was performed at 90 volts for 30 minutes with the EDVOTEK tetra source electrophoresis machine, Bethesda, USA. The targeted genes (HIV-1 GAG) were visualized by Wealtec Dolphin – DOC UV transilluminator and photographed. The molecular weights were calculated using molecular weight standard of the marker.

2.6. Statistical Analysis

The data obtained from the study was analyzed using SPSS version 25.0 statistical package. The results were expressed as mean ± standard error of mean (SEM). Statistical difference between groups were done using Analysis of variance. The difference was considered significant at $P \leq 0.5$.

3. Results

The results obtained in the study are presented in the table 1 to 2 and plate 1 to plate 2

3.1. Characteristic of the Study Population

The characteristics of the study population (mean age) are shown in table 1. The values of age in the subjects group A; TDF + 3TC + EFV (46.9years), group B; AZT + 3TC +NVP (35.8years) were significantly higher ($p<0.05$) than similar value in the controls (37.0years).

3.2. Mean serum levels of P24 antigen and HIV-1protease in subjects on TDF + 3TC + EFV, AZT + 3TC + NVP and controls.

the values of P24 antigen and HIV-1 protease are shown in table 2, the values in the subjects on TDF + 3TC + EFV (9.64ng/ml and 5.48mU/mg), AZT + 3TC + NVP (13.01 ng/ml and 20.47mU/mg) were significantly lower ($p<0.05$) than similar values in the controls (16.3ng/ml and 26.68mU/mg)

Table 1 Characteristics of the Studied Population (SEM)

Subjects	N	Age(Years)
Group A (TDF + 3TC + EFV)	25	46.9 ± 0.74
Group B (AZT + 3TC +NVP)	25	38.8 ± 0.85
Controls (those not on drugs)	25	37.0 ± 0.83
P value		P = 0.04*

Values differ significantly from controls ($p<0.01$); n = sample size; SEM = standard error of mean; TDF = Tenofovir; 3TC = Lamivudine; EFV = Efavirenz; AZT = Zidovudine; NVP = Nevirapine

Table 2 Biochemical parameters for the subjects and controls

Subjects	N	p24(ng/ml)	Protease (mU/mg)
Group A	25	9.64 ±5.34	5.48 ± 6.62
Group B	25	13.01 ± 5.19	20.47 ± 7.53
Group C	25	16.30 ±3.68	26.08 ± 11.71
F-Value		12.57	19.45
P- value		0.001*	0.001*
AVB		0.211(ns)	0.100(ns)
AVC		0.001(s)	0.001(s)
BVC		0.001(s)	0.001(s)

Group A = (TDF + 3TC + EFV); Group B = (AZT + 3TC +NVP); Group C = (those not on drugs); ns = not significant; s = significant

3.3. HIV-1 POL and GAG Gene Expression

3.3.1. Figure 1

HIV-1 RNA RT – PCR product of POL genes:

HIV-1 RNA extraction and POL RT – PCR was done on 10 samples from the studied population with elevated P24 antigen and elevated protease (2 samples from group A, four samples from group B and four samples from group C). L_1 and L_{12} is a 100bp DNA ladder as shown on the plate 1, lanes L_2 and L_3 are positive bands expression of HIV-1 POL at 100bp of two subjects from group A, L_4, L_5, L_6, L_7 are positive bands expression of HIV-1 POL at 100bp of four subjects from group B, while L_8, L_9, L_{10}, and L_{11}, bands are positive bands expression of HIV-1 POL at 100bp of four subject from group C. The bands of lane L_2, L_3, L_{10}, and L_{11} bands are moderately faint due to low POL – genes concentration in the RNA template; though the protease enzyme may be elevated from the subjects in L_2, L_3, L_{10}, L_{11}; the reverse transcriptase and integrase

enzymes may have contributed to the low POL- genes expression in the template. Lanes L_4, L_5, L_6, L_7, L_8, and L_9 are boldly expressed due to high concentration of protease enzymes; contributed also from reverse transcriptase and integrase enzymes.

Figure 1 Electrophoregram of POL-genes of the HIV-1 RNA RT-PCR PRODUCTS. Sample; 100bp PCR Product, Ladder; 100bp, 250bp and 500bp.

3.3.2. Figure 2

HIV-1 RNA GAG PCR analysis was done on 10 samples as shown in plate 2 from studied population with elevated p24 antigen and elevated HIV-1 protease (2 samples from two subjects in group A, four samples from four subjects in group B and four samples from subjects in group C).

The bands B_1 and B_{12} is a 100 – 500bp DNA as shown in plate 2, B_2, and B_3 are bands expressions of HIV-1 GAG at 100bp from two subjects in group A, bands B_4, B_5, B_6, and B_7, are GAG band expressions from four subjects in group B, while bands B_8, B_9, B_{10}, and B_{11}, are GAG expressions of four subjects in group C at 100bp. Bands B_2, B_3, B_4, B_5, B_6, and B_{10}, are faintly expressed GAG of HIV-1, while bands B_7, B_8, B_9 and B_{11} are negatively expressed. The weakly or faintly expressed GAG and negatively expressed GAG in HIV-1 RNA could be due to drops in concentration of P24 antigen, Nucleocapsid (NC), and the matrix proteins (P17) of the HIV-1 in chronic condition.

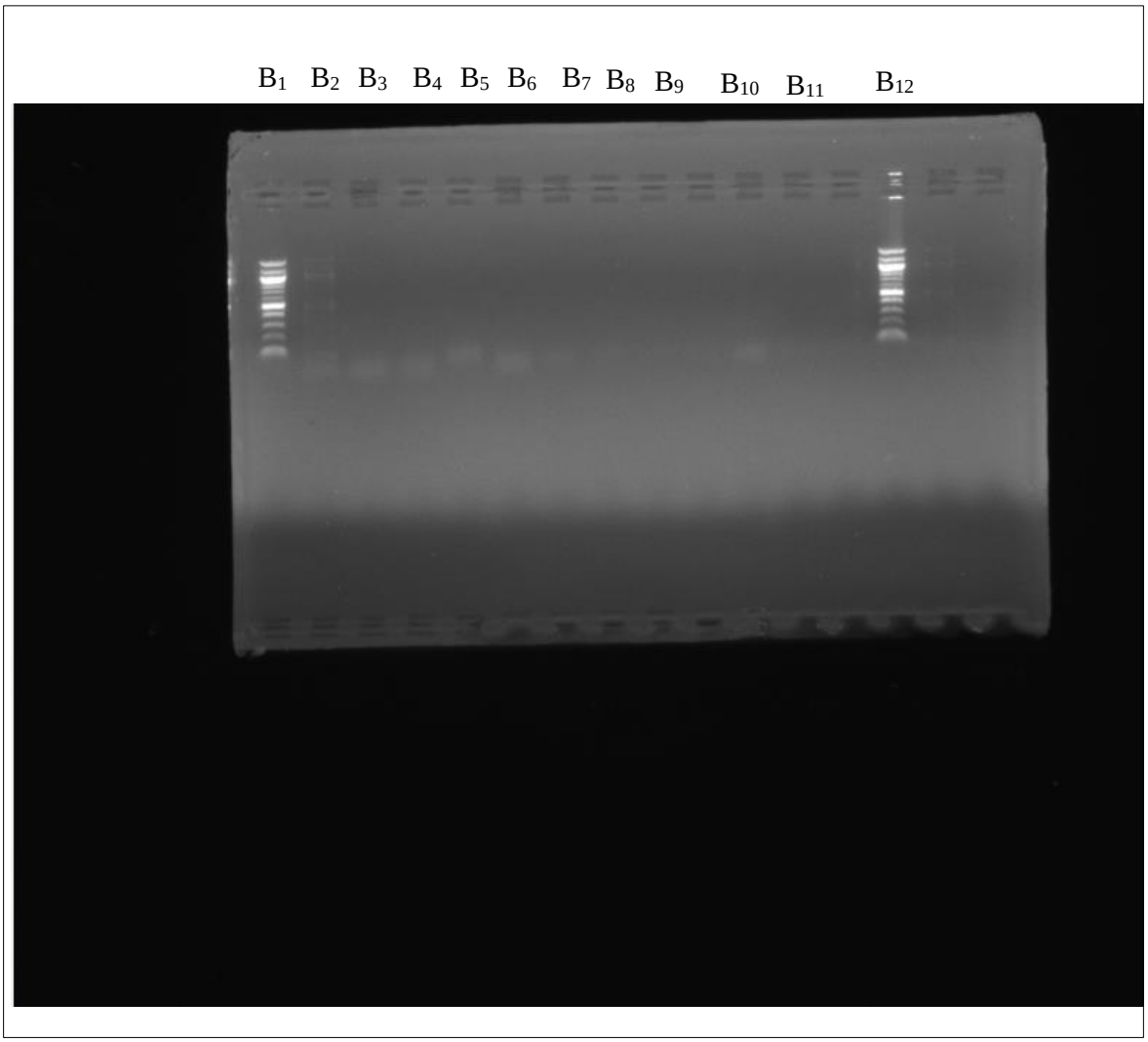

Figure 2 Eletrophoregram HIV-1 RNA GAG-PCR Product.

Sample; 100bp PCR product, Ladder; 100bp, 250bp and 500bp.

4. Discussion

This study observed that the mean serum values of P24 antigen was significantly lower in subjects on TDF + 3TC + EFV when compared with subjects on AZT + 3TC + NVP. Also noted is the HIV-1 protease which showed lower activity for subjects on TDF + 3TC + EFV when compared with subjects on AZT + 3TC + NVP. The significant higher value of P24 antigen and HIV-1 protease for subjects on AZT + 3TC + NVP closely to that of controls showed that this group of drugs lacks treatment efficacy compared to TDF + 3TC + EFV.

The pol (polymerase) codes for P66 and P51 subunits of reverse transcriptase and P31 an endonuclease; located in the core, close to nucleic acids [17]. The Pol is responsible for conversion of viral RNA into cDNA, integration of cDNA into host cell DNA and cleavage of protein precursors [5].

From the Pol expressions, almost all the samples were expressed; this shows that Pol-gene expression is a good marker for monitoring of patients on antiretroviral treatment in the detection of treatment failure when compared to Gag with faint or negative expressions. This study findings is in agreement with the study of Rawizza *et al.,* [23] that says the levels of Gag-Pol protein expression can correlate with disease progression, influencing treatment decisions and prognostic assessment. The findings are supported by Miranda *et al.,* [18] that maintenance of Gag/pol ratio is important for HIV-1 dimerization and viral infectivity. Miranda *et al.,* [18] showed that there was alteration in the expression of Gag/pol ratio at advanced stage of the HIV/AIDS disease changing the normal Gag-pol ratio from 20:1 to 20:21; this means over expression of pol genes with reduced expression of Gag genes. The authors maintained that the over

expression of pol genes could be due to excess inactive protease enzyme (PR-) in the presence of few active protease enzyme (PR+) [18].

The low active protease enzyme (PR+) could be responsible for reduced proteolytic cleavage of Gag precursor and this may probably lead to low or negative Gag genes expression [18]. From these findings; pol expression at advanced stage of HIV/AIDS disease correlate better with treatment failure than Gag genes expression; this support the findings of Johnston *et al.,* [11].

Gag (group specific antigen); is located in the nuclocapsid of virus-made up of P24 and P15, P17, P55, P7 and P9 [4, 6]. Both Gag and Pol are components of HIV structural genes including envelope (ENV) gene [13]. In chronic stage of the disease, the increase in antibody could decreases the P24 antigen; this might account for the negative expression of Gag genes [18]. Understanding the dynamics of Gag-Pol expression can help optimize combination therapies, which are more effective in suppressing viral load and preventing resistance.

5. Conclusion

Quantification of P24 Antigen, HIV-1 protease and HIV-1 Gag-Pol genes expression are advocated for in resource limited settings as an alternative to viral load which is often not available in most African health facilities. The increase in P24 antigen and HIV-1 protease is an indication of treatment failure with broadly expressed RNA Pol and Gag genes. The focus in resource-limited settings has been almost exclusively on increasing access to antiretroviral drugs; attention must now be paid to laboratory monitoring to limit the costs associated with the widespread use of expensive second-line therapy and to provide optional monitoring tools for participants. Further longitudinal study using HIV-1 protease, HIV-1 p24 antigen and HIV-1 Gag-Pol gene expressions is also necessary to ascertain a clearer picture of subjects on HAART with treatment failure or success.

Compliance with ethical Standards

Acknowledgement

Authors wish to acknowledge all the HIV-1 infected individuals who voluntarily agreed to participate in this study.

Disclose of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The ethical approval was obtained in accordance with the principles of declaration of Helsinki from the board of ethics committee of Kogi State Ministry of Health, Kogi State, Lokoja, Nigeria.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study

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