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Molecular Detection of Human Immunodeficiency Virus from Healthy Blood Donors at Two Tertiary Hospitals in Nigeria

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Abstract Introduction**:** Human Immunodeficiency virus (HIV), a retrovirus and enveloped RNA virus is one of the infectious agents been tested before a blood donor is qualified for donation based on its negativity. The aim of the study was to detect and identify HIV genotypes of healthy blood donors certified fit for donation at University of Abuja Teaching Hospital (UATH) Abuja, Nigeria and Nnamdi Azikiwe University Teaching Hospital (NAUTH) Nnewi, Nigeria. **Methodology:** Blood samples from 197 apparently healthy blood donors (98- UATH and 99-NAUTH) were screened using rapid test kit (Determine HIV Kit). All samples that were negative by rapid test kit were retested using 4th generation HIV ELISA (Fortress Diagnostics UK). Twenty two positive and negative samples by ELISA each were tested using nested RT-PCR to detect HIV RNA. Eight samples positive for HIV RNA were amplified and V3 gene sequenced to identify HIV genotypes. **Results:** Out of 197 samples that tested negative by rapid test, 27 (13.7%) were positive by ELISA. Twenty two samples negative by HIV ELISA were negative for HIV RNA and 8 (36.4%) of 22 samples positive by ELISA were positive by HIV RNA. Of the 8 HIV RNA positive samples sequenced, 4 (50%) were successfully sequenced. The DNA sequencing and phylogenetic analysis showed that the isolates sequenced belonged to HIV-1 with gene sequences similar to HIV isolates from Cameroon, Senegal and Italy. **Conclusion:** High prevalence of HIV infection among blood donors that tested negative by rapid test kit signified that blood recipients could be transfused with infected blood units unknowingly testing for anti-HIV. HIV type 1 is the most prevalent genotype among the blood donors with gene sequences similar to isolates from Cameroon, Italy and Senegal. It is recommended that 4th generation HIV ELISA be used for screening blood donor to reduce HIV transfusion risk.

Keywords: *HIV genotypes, blood donors, gene sequencing*

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1. Introduction

Human Immunodeficiency virus (HIV) is a retrovirus, an enveloped RNA virus, which is transmissible by the parenteral route. It is one of the transfusion transmissible agents been tested before a blood donor is qualified for donation based on its negativity [1] It is found in blood and other body fluids. Once in the bloodstream, the virus primarily infects and replicates in lymphocytes. The viral nucleic acid persists by integrating into the host cell DNA. A number of different groups and subtypes (clades) have been identified with some significant antigenic differences. HIV-1 and HIV-2 are the two major distinct virus types and there is significant cross-reactivity between them. HIV-1 is now endemic in many parts of the world, although its incidence and prevalence is low in some regions. HIV-1 group M is responsible for more than 99% of the infections worldwide, whereas the prevalence of HIV-2 is mainly restricted to countries in West Africa and India. Additionally, a few infections with HIV group O and group N have been observed in Africa. The appearance of antibody marks the onset and persistence of infection, but not immunity. As HIV can be present in the bloodstream in high concentrations and is stable at the temperatures at which blood and individual blood components are stored, the virus may be present in any donated blood from an HIV-infected individual. Infectivity estimates for the transfusion of infected blood

products are much higher (around 95%) than for other modes of HIV transmission owing to the much larger viral dose per exposure than for other routes [2].

The screening methods used to identify the presence of HIV employ the following screening targets: Serological markers (anti-HIV-1, including group O, plus anti-HIV-2, HIV p24 antigen (p24 Ag) and Viral nucleic acid: HIV RNA [3]. The assay should be capable of detecting subtypes specific to the country or region. Screening donations for both antibody and antigen will identify the vast majority of donations from infected donors [4]. This entails the use of Anti-HIV-1 plus anti HIV-2 and p24 antigen. All screening strategies should employ, at minimum, the detection of antibody because the identification of specific antibody is still the most reliable screening method. They should preferably also employ the detection of antigen. Antibody to HIV may be detected approximately three weeks after infection and approximately six days after antigen is first detected [5]. Human Immunodeficiency Virus (HIV) p24 antigen may appear from 3 to 26 days after viral RNA [6], and its detection can further reduce the serological window period by 3 to 7 days before antibody detection. Screening for anti-HIV has been the basis for blood screening since the mid-1980s and HIV serology is therefore well understood. Although there is cross-reactivity between the main virus types (HIV-1 and HIV-2), it is not sufficient to rely on an HIV-1 specific assay to detect all cases of HIV-2. Since the early 1990s, anti-HIV assays have included specific antigens for both HIV-1 and HIV-2. However, the use of antibody-only assays has been superseded by the use of combination HIV antigen and antibody assays (combined HIV p24 Ag and anti-HIV-1 + anti-HIV-2), wherever possible. These provide an enhanced level of sensitivity in early infection over antibody-only assays by reducing the serological window period [7]. Moreover, viral RNA can be detected approximately 7 to 11 days after infection: i.e. when the results of HIV antigen-antibody assays are negative, but HIV RNA detection is positive. The detection of HIV RNA can reduce the risk of HIV being transmitted through the transfusion of infected blood donated during the serological window period of antigen and antibody assays. To minimize the risk of HIV infection through the route of transfusion, screening should be performed using a highly sensitive and specific anti-HIV-1 + anti-HIV-2 immunoassay or HIV combination antigen antibody immunoassay (EIA/CLIA). The assay should be capable of detecting subtypes specific to the country or region. In addition, screening using a highly sensitive and specific anti-HIV-1 + anti-HIV-2 rapid assay may be performed in laboratories with small throughput, in remote areas or emergency situations.

In most blood banks in Nigeria including our study centers, screening of blood donors is only done with rapid test kits that detect HIV antibodies. Blood donor screening is rarely done using ELISA and Nucleic Acid Tests (NATs) to detect HIV antigen and HIV RNA. This means that blood donors at the window period of infection may be missed as antigen/viral RNA was not tested. There is paucity of data on molecular detection of HIV and their genotypes among blood donors in our study population. This study was embarked upon to compare the efficacy of rapid test kit that detects HIV antibodies, 4th generation ELISA that detects both antibodies and p24 antigen and viral RNA to detect HIV infection among previously screened blood donors at NAUTH, Nnewi and UATH Abuja. The study also sequenced the V3 gene of HIV isolate to identify the genotype. This will help in making evidence based recommendations to improve blood donor selection and screening. The identification of HIV genotype and phylogenetic relationship of isolates to other HIV isolates deposited at National Centre for Biotechnology Information (NCBI) using gene sequencing and phylogenetic analysis will be useful for molecular epidemiological studies.

2. Materials and Methods

2.1. Study Sites

The study sites comprise the blood banks of University of Abuja Teaching Hospital (UATH) Gwagwalada, Abuja and Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi from Northern and Southern Nigeria respectively.

2.2. Subjects

These include 197 (98 from UATH and 99 from NAUTH) apparently healthy blood donors who had been previously screened and found eligible by the respective blood banks for donation. They were recruited over a period of five months, from June 2016 to October, 2016. Relevant Sociodemographic information and risk factors of HIV infection were obtained from blood donors using structured questionnaire.

2.3. Ethical Considerations

Ethical permission was obtained from the research ethics committee of both hospitals and informed consent was obtained from the subjects before sample collection.

2.4. Study Design

This is a descriptive, cross-sectional, laboratory based study conducted at Nnamdi Azikiwe University Teaching Hospital, Nnewi and University of Abuja Teaching Hospital, Gwagwalada Abuja, Nigeria.

2.5. Specimen

Ten milliliters of venous blood samples were taken from each blood donor; 5ml into a clean dry plain tube and 5ml into an EDTA tube. Blood samples in plain tube were allowed to stand at room temperature for clotting and retraction. Thereafter, the samples were centrifuged to give a clear serum. Sera were stored at -20°C until various tests were performed while whole blood in EDTA was preserved at 2-6°C before testing.

2.6. Serological Analysis

2.6.1. Determination of HIV-1/2 Antibodies using Determine HIV Rapid Kit

Alere Determine HIV-1/2 is an immunochromatographic test for the qualitative detection of antibodies to HIV-1

and HIV-2. Sample is added to the sample pad. As the sample migrates through the conjugate pad, it reconstitutes and mixes with the selenium colloid-antigen conjugate. The mixture continues to migrate through the solid phase to the immobilized recombinant antigens and synthetic peptides at the patient window site. If antibodies to HIV-1 and/or HIV-2 are present in the sample, the antibodies bind to the antigen-selenium colloid and to the antigen at the window forming a red line at the patient window site. If antibodies to HIV-1 and /or HIV-2 are absent, the antigen-selenium colloid flow past the patient window and no red line is formed at the patient window site. Assay procedures as described by the manufacturer were strictly followed.

2.6.2. HIV Testing by ELISA

The samples were screened for HIV using Fortress Diagnostics 4th generation ELISA kit and read with Stat Fax 2100 Plate Reader. The HIV ELISA test kit used for this study has the capacity to detect anti-HIV and p24 antigen. Manufacturer's instructions were strictly followed in performing the assay.

2.7. Determination of Some Haematological Parameters Using Sysmex Auto-haematology Analysis

Complete blood count of blood donors positive for HIV and those negative for HIV was done using Sysmex KN 21 auto hematology analyzer. The Sysmex KN 21 auto-hematology analyzer used was able to determine the hematological indices of the blood donors like HGB, RBC, WBC total and differential, HCT and Platelets. The Sysmex KN 21X Auto-hematology analyzer is a quantitative, automated hematology analyzer and leucocyte differential counter for in-vitro diagnostic use in clinical laboratories. The tests were done according to the instruction of the automatic multiparameter blood counter.

2.8. Determination of CD4 Cell Count Using Cyflow Counter

The CD4 cell count was performed on blood donors positive and negative for HIV following the method as described by [8]. This was done using Cyflow counter machine by Partec Germany. Flow cytometry is a method by which cell or micro particles in suspension is differentiated and counted according to the cell size, fluorescence emission and internal structure.

2.9. Molecular Studies

2.9.1. RNA Extraction from Plasma Samples

RNA extraction was performed according to [9] using Zymo extraction kit (Zymo Research Corporation) and marketed by Ingaba Biotech Ltd Pretoria, South Africa. RNA was extracted from HIV negative/positive samples and then PCR was used for detection of HIV-RNA. The extraction was done according to the guidelines of the manufacturer.

2.9.2. Amplification of HIV V3 Region (Nested PCR)

Conventional nested RT-PCR for the detection of HIV RNA was performed with normal ABI *Taq* polymerase as described by [10] The PCR amplified products were electrophoresed in 2% agarose gel stained with ethidium bromide and observed under gel documentation system (Bio-Rad, USA).

2.9.3. Deoxyribonucleic Acid Sequencing and Phylogenetic Analysis

Four PCR-positive samples (HIV RNA positive) were successfully sequenced. The V3 gene portion of HIV was successfully sequenced. The DNA sequencing was done using Sanger sequencing method as described by [11]. The PCR purified products were directly used for sequencing in the BigDye Terminator cycle sequencing kit and analyzed on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. Gene Sequencing was performed according to manufacturer's instructions.

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbour-Joining method in MEGA 6.0 [12]. The bootstrap consensus tree inferred from 500 replicates [13] is taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method [14].

2.10. Statistical Analysis

Data obtained from this study were entered on Microsoft excel, validated on SPSS version 20 for windows and analyzed on Epi Info version 7 for windows. Also Graph pad prism software version 7.0 was used for some data analysis. Descriptive statistics (frequency, means, and standard deviation) was used to estimate participants' socio-demographic characteristics at 95% confidence interval. Inferential data analysis such as Chi-Square was used to establish the association/relationship between two variables. Statistical significance was determined at a probability level of $p< 0.05$.

3. Results

Comparison of prevalence and detection of HIV Infection among blood donors using rapid test kit and ELISA is presented in Table 1. The data obtained showed that out of 197 samples tested for HIV infection using rapid test kit (Determine Kit), none was positive for HIV antibodies while 27 (13.7%) samples out of 197 were positive for HIV by ELISA. There is statistical significant difference observed in the use of these methods to detect prevalence of HIV infection in the study population.

Table 2 presented the prevalence of HIV RNA among blood donors that tested negative and positive by ELISA. The result showed that all samples (22) that tested negative to HIV ELISA were also negative for HIV RNA while 8 (36.4%) of 22 samples that tested positive by HIV ELISA were positive for viral RNA.

Table 1. Detection of HIV Infection among Blood Donors Using Rapid Test and ELISA in the Study Population

Assay format	No of Samples Tested	No (%) of Positive Samples	No (%) of Negative samples	Chi-Square (p-value)
Rapid Test	197	0 (0%)	197 (100%)	*50.49 (<0.0001)**
ELISA	197	27 (13.7%)	170 (86.3%)	

*Statistically significant (p< 0.05).

Table 2. Prevalence of HIV RNA among Blood Donors that Tested Negative and Positive by ELISA Technique in the Study Population

ELISA	No of Samples of Samples Tested	No. (%) Positive for HIV RNA
Positive	22	8 (36.4%)
Negative	22	0 (0%)

Table 3. Mean Comparison of CD4 and Haematological Parameters of HIV Seropositive and HIV Seronegative Blood Donors in the Study Population

Haematological Parameters	HIV Seropositive Blood Donors n= 22 X±SD	HIV Seronegative Blood Donors n= 22 x±SD	Student-t Test	p-value
CD4	779±28	926±30	1.37	*0.25*
WBC	5.1±2.6	5.0±2.2	0.18	*0.67*
HGB	11.9±3.5	13.1±3.6	1.41	*0.24*
HCT	38.3±6.2	38.2±6.2	1.13	*0.29*
PLT	237±15	205±14	1.41	*0.24*
RBC	4.79±2.19	5.06±2.25	1.53	*0.22*

Statistically significant (p< 0.05)
Key: RBC: Red blood cells, HCT: Haematocrit, HGB: Haemoglobin, WBC: White blood cells, CD4: Cluster of differentiation type 4, PLT: Platelets.

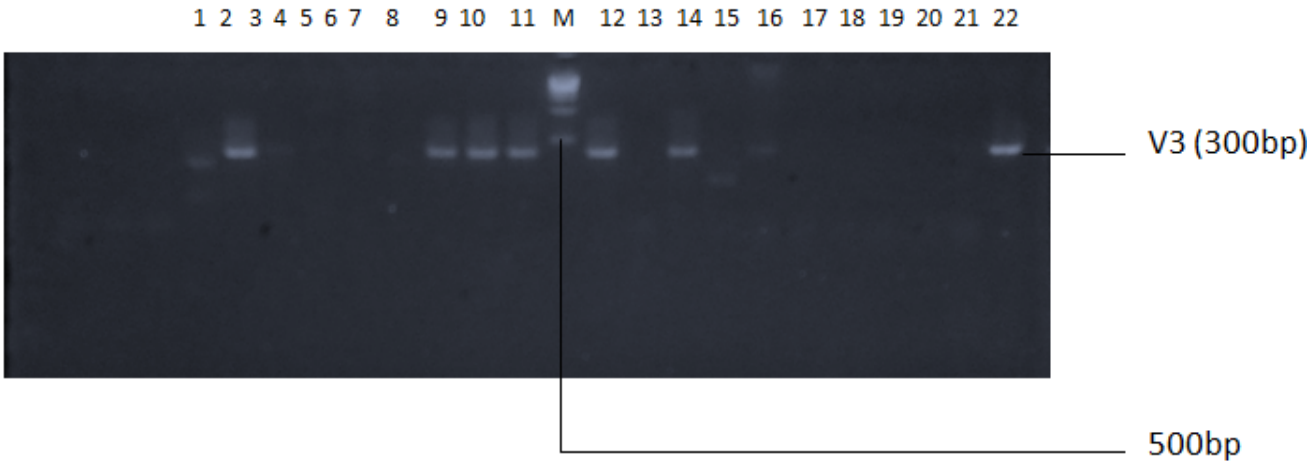

Plate 1. Agarose gel electrophoresis showing the V3 bands of the HIV among blood donors in the Study Population. M represents the 1kb molecular ladder, while lanes 2,9,10,11,12,14 and 22 represent the Amplified V3 region

Table 3 shows mean comparison of CD4 and Haematological Parameters of HIV Positive and HIV Negative Blood Donors in the Study Population. On the average there was no statistical significant different between these 2 groups of blood donors. The hematological values of HCT, HGB, WBC, RBC, Platelets count and CD4 Cell count fall within the normal acceptable range.

4. Gene Sequencing and Phylogenetic Analysis Results

The sequences were analyzed by using BioEdit 9.7 and Codon-code Aligner 4.0 software. Sequence analysis and comparison were conducted by using molecular programs deposited in the web site of the National Centre for Biotechnology Information (NCBI). The sequences were compared with the same region of HIV sequences from different genotypes found in the genotyping reference set available on the NCBI website. The phylogenetic tree was constructed according to previous methods [15]. Four (50%) out of 8 HIV isolates were successfully sequenced. The V3 gene of HIV isolates was sequenced. The sequencing result shows that the HIV isolates were all HIV-1. The figures below show the phylogenetic trees of HIV isolates in relation to other isolates matched with them to show their relatedness based on gene sequences.

The obtained V3 sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The V3 the isolate P1 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P1 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate P3599 (gb: DQ32363.1) than the V3 of other HIV viruses (Figure 1). The V3 the isolate P2

showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P2 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate NGIB 04_009 (gb: KF437610.1) than the V3 of other HIV viruses (Figure 2). The obtained V3 sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database.

The V3 the isolate P3 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P1 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate MFU54_D7 (gb: HQ236565.1) than the V3 of other HIV viruses (Figure 3). The obtained V3 sequence from the isolates produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database.

The V3 the isolate P4 showed a percentage similarity to other species at 99%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the V3 of the isolate P4 within the HIV viruses and revealed a closely relatedness to the V3 of HIV-1 isolate 025P34778/02 (gb: AY304381.1) than the V3 of other HIV viruses (Figure 4).

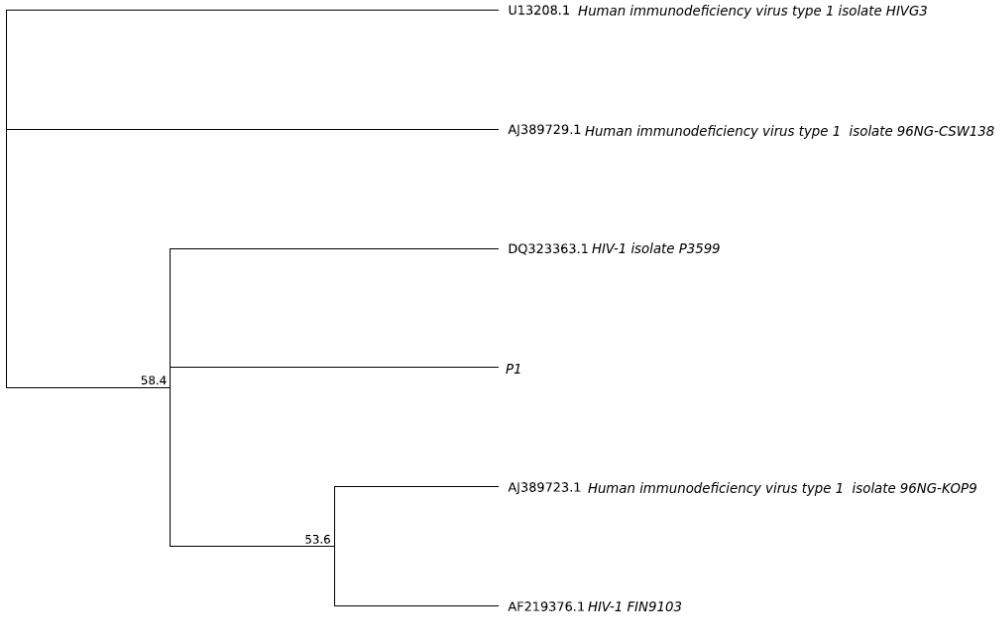

Figure 1. Phylogenetic Tree showing Relationship between the V3 of the isolate P1 from a Blood Donor and the V3 of other HIV isolates

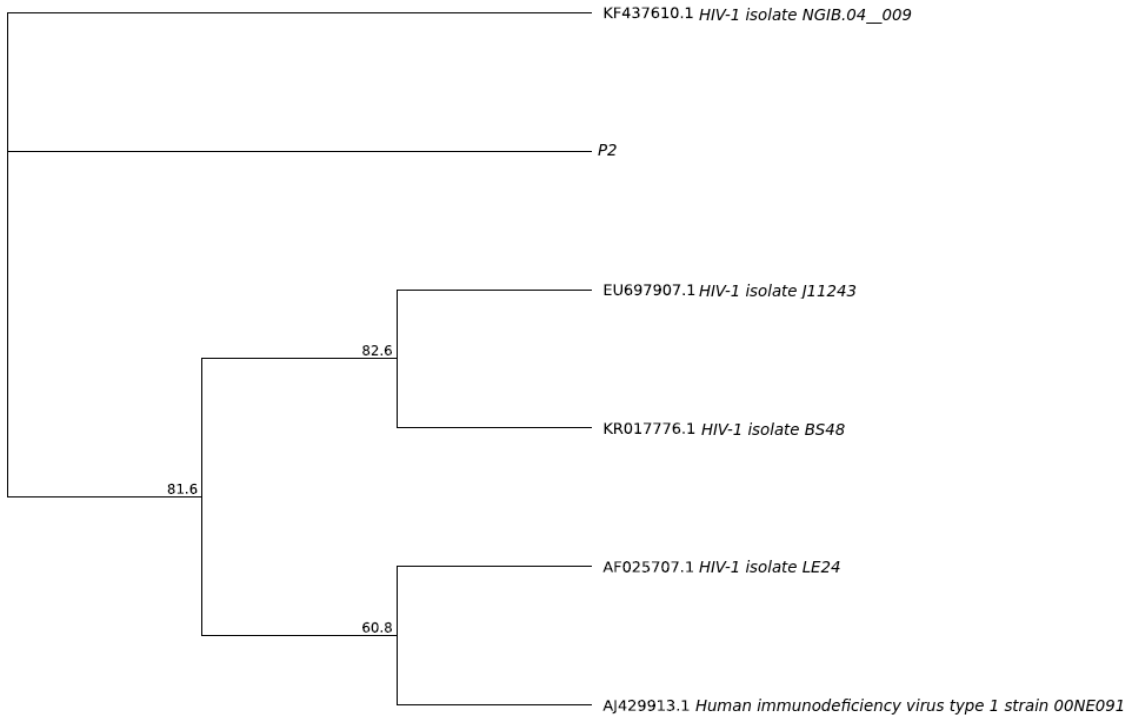

Figure 2. Phylogenetic Tree showing Relationship between the V3 of the isolate P2 from a Blood Donor and the V3 of other HIV isolates

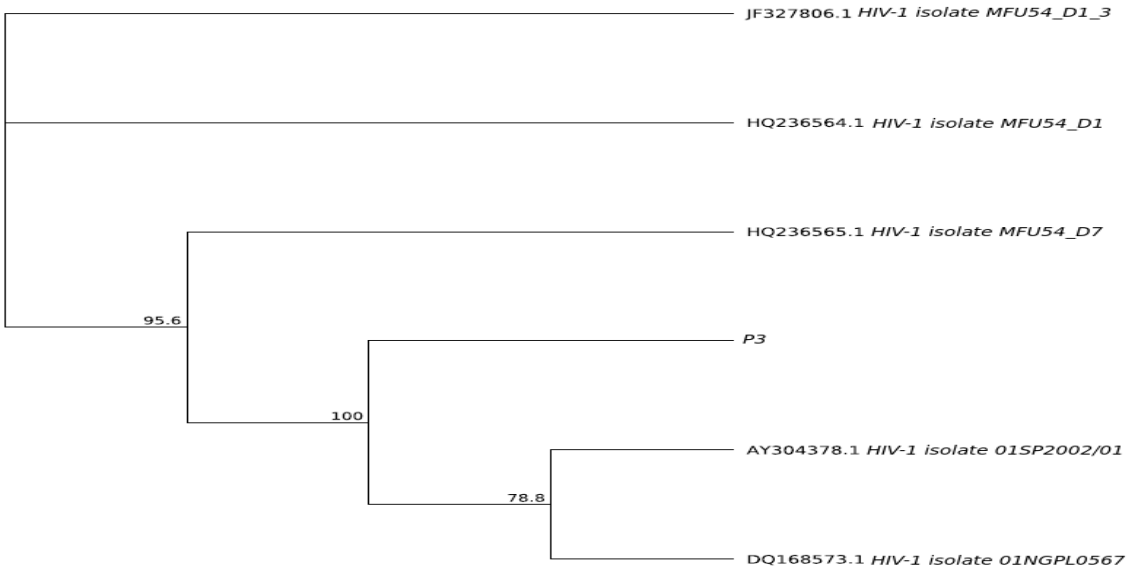

Figure 3. Phylogenetic Tree showing Relationship between the V3 of the isolate P3 from a Blood Donor and the V3 of other HIV isolates

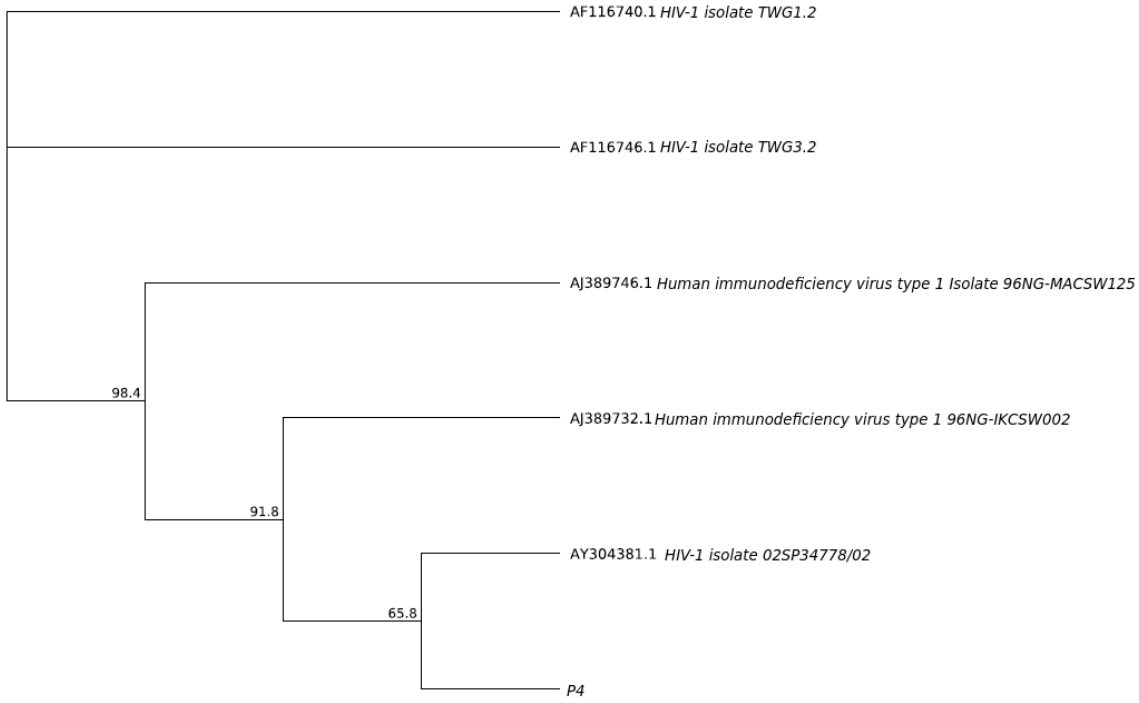

Figure 4. Phylogenetic Tree showing Relationship between the V3 of the isolate P4 from a Blood Donor and the V3 of other HIV isolates

5. Discussion

The ELISA results of blood donors tested for HIV infection in the study population showed that out of 197 samples that tested negative to rapid test kit 27 (13.7%) were positive with 4th generation ELISA kit. These values were quite high and significant. The reason for this high positivity rate could be as a result of highly sensitive ELISA HIV 4th generation kit used in this study. This HIV ELISA 4th generation kit contains antibody and antigen (p24) making it more sensitive in detection of small concentration of HIV in the blood specimen [16] compared to antibody based assay (rapid test kit) used in screening blood donors in the study population. This indicates that ELISA technique containing antibody and p24 antigen is more sensitive and superior than rapid test for screening of blood donors for HIV infection. Failure of the rapid kits to detect the presence of markers of infectious viral diseases may be due to inadequate coating of the antigen, nature of the antigen used and genetic heterogeneity of the virus [17,18]. In this study we observed high false negative results with the rapid diagnostic kit compared to ELISA. Our finding is in agreement with previous report of [19] which indicated that there is risk of donor blood samples containing HBV being transfused to patients due to suboptimal testing using HBsAg rapid kits only. This means that blood units screened with rapid test kit is able to transmit HIV infection when transfused to blood recipients.

The results obtained from ELISA testing for HIV showed that ELISA format is more sensitive than rapid test kit [20] but PCR is also highly sensitive and specific

in detection HIV RNA of this infectious agent. This also signifies that the rapid test kits being used for HIV screening in the blood banks where the subjects were recruited were not adequate for screening in blood transfusion services and would allow transfusion of HIV positive blood in at least 10 out of every 100 blood donations. The inadequacy in sensitivity and variation in performance between different locations of some rapid test kits used in resource poor settings for HBsAg screening has been shown by [21]. In their cross-sectional epidemiological study they compared the accuracy of rapid test immunochromatographic kits in the detection of HBsAg, anti-HBc and anti-HCV against ELISA, in two populations of 1200 potential blood donors in rural Cambodia and Vietnam. For HBsAg specifically, they found the rapid test kits to be high in specificity (99.8–99.9 %) but lower in sensitivity (86.5 %). They also found a difference in its sensitivity between both countries. In Cambodia the sensitivity was 93.5 % and in Vietnam 81.8 %. These collaborate with this study that showed that ELISA is more sensitive than rapid test in detection of HIV infection.

This study also observed that all HIV isolated from blood donors belonged to HIV-1.This finding is in accord with the report of [16] that found HIV-1 most prevalent subtype reported globally. The phylogenetic analysis as seen in Phylogenetic trees developed showed that HIV isolates from this study have close relatedness with isolates from Senegal, Cameroon and Italy and all isolates belonged to HIV-1 [22]. This could be as result of intra border transfer of these viruses from one country to another. Moreover, the HIV isolates from UATH Abuja and NAUTH Nnewi are similar. This could be as result of people moving from state to another part of Nigeria and in the process spread this virus from one person to another as HIV can be transmitted through various modes. This include through sex, needle prick injuries and visiting commercial barbing saloon to mention but a few [23].

The results of haematological parameters and CD4 cell count of HIV seropositive and HIV seronegative blood donors showed that there is no statistical significant increase between the blood values and CD4 count of HIV seropositive and seronegative. This result is possible because the blood donors infected with HIV are healthy and the viral load may not be high to extent to damage the blood cells. However, it is important that these blood donors be enrolled in HIV treatment with antiretroviral drugs to forestall future alteration and destruction of blood cells and some organs of the body. We were unable to perform HIV viral load because of limited fund. This calls for further study.

6. Conclusion/Recommendations

There is a high prevalence of HIV infection among blood donors previously screened with rapid test kit and certified fit for donation. HIV-1 is the most prevalent genotype and phylogenetic analysis showed that the gene sequences are similar to isolates from Senegal, Cameroon and Italy, indicating great diversity of HIV genotype among blood donors in the study population. Further study is needed to determine the viral load and detect mutation in the genes of HIV isolates. The study hereby recommends that blood donors in these hospitals in particular and Nigeria in general be screened with ELISA 4th generation kit as it is highly sensitive. Though it may lead to more rejection of blood donors/blood units, it will enhance the safety of blood recipients.

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Conflicting Interests

The authors declare that they have no conflicting interests.

Authors' Contributions

AIO conceived the study. AIO, AD and MOI were in charge of recruitment of blood donors and collection of samples. AIO, AOC, AD and BMM undertook laboratory analysis; AIO and NRA analyzed the data and prepared the manuscript; NRA, MOI BMM and AOC provided ideas and comments during manuscript preparation. All authors have read through and approved the final manuscript.

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