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Research Article

Detection of HIV-1 CRF35_AD among Multiple Strains of Circulating HIV-1 Subtypes in Ibadan, Nigeria

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Abstract
Molecular analyses of Human Immunodeficiency Virus (HIV) in Nigeria have shown the presence of multiple strains of the virus in circulation with ongoing virus diversification over time. This study was carried out to determine the circulating HIV-1 strains, and their year to year changes over a three-year period. Blood samples were collected from 85 consenting HIV-1 infected anti-retroviral therapy-naïve patients. They include 42 males and 43 females with median age of 37 years (range 18-58 years) who presented at a voluntary counseling and testing centre in the University College Hospital, Ibadan. The env C2-V3 region of HIV-1 from the genomic DNA extract was amplified by nested PCR using primers WT1 and WT2, and KK40 and KK30 for the first and second rounds, respectively, and directly sequenced in both forward and reverse directions, edited and analysed for phylogenetic relationships by ClustalW and Maximum Likelihood methods in MEGA 5.03 software based on their nucleotide sequences. The target region was successfully sequenced from 64 HIV positive patients and identified as subtype A (3.13%), G (53.13%), CRF02_AG (28.13%), CRF06_cpx (14.06%), and CRF35_AD (1.56%) with variation noticed in subtype distribution over the three years of the study.

Keywords: HIV-1 strains, Genetic diversity, C2-V3 region, CRF35_AD

INTRODUCTION

The global distribution of HIV-1 is complex and dynamic with continental, sub-continental, national and regional epidemics harbouring only a subset of the global diversity (McCutchan, 2006). The increasing virus diversification, which is maintained and sustained through mutation and recombination, has resulted in the classification of HIV-1 into 4 phylogenetic groups M, N, O and P (Hemelaar, Gouws, Ghys, & Osmanov, 2006; McCutchan, 2006; Plantier *et al*., 2009). The Group M consists of at least 9 different subtypes (A, B, C, D, F, G, H, J, and K), over 55 circulating recombinant forms (CRFs) and many unique recombinant forms (Delgado *et al*.; Fernandez-Garcia *et al*., 2010) .

Africa, in particular West-Central Africa, shows the greatest molecular diversity or heterogeneity of HIV-1 and they are associated with the largest proportion of HIV infections worldwide (Hamel *et al*., 2007; Ishikawa *et al*., 1996; Kanki *et al*., 1999; Powell, Barengolts, Mayr, & Nyambi; Sankale *et al*., 2007)). In Nigeria multiple subtypes and CRFs of HIV-1, and HIV-2 (Olaleye, Ekweozor, Sheng, & Rasheed, 1995; Olaleye *et al*., 1993) have been reported from studies conducted in different parts of the country and they include subtypes A, A1, A2, B, C, D, G, G', CRF01_AE, CRF02_AG, CRF06_cpx, CRF09_cpx and CRF11_cpx (Abimiku *et al*., 1994; Agwale *et al*., 2002; Ajoge *et al*., 2011; Chaplin *et al*.; Howard, Olaleye, & Rasheed, 1994; Odaibo *et al*., 2006; Ojesina & Kanki, 2006; Ojesina *et al*., 2006; Peeters *et al*., 2000; Sankale *et al*., 2007).

The continual surveillance of HIV subtypes and their phylogenetic relationships helps to identify outbreaks that are possibly caused by newly introduced variants, and, in turn, this information can help in planning of prevention programs, and can potentially contribute to halting the onward transmission of a particular outbreak. This study was therefore carried out to characterize the circulating strains of HIV-1 among HIV-1 positive individuals and to assess their year to year variation..

MATERIALS AND METHODS

Blood samples were collected from 85 consenting HIV-1 infected anti-retroviral therapy-naïve individuals. They include 42 males and 43 females with median age of 37 years (range 18-58 years) who presented at a voluntary counseling and testing centre in the University College Hospital, Ibadan from January 2004 to December 2006. The University of Ibadan/UCH ethical review board approved the study protocol and written informed consent was obtained from every individual whose blood sample was used for the study.

Blood sample was collected by venepuncture from each patient into sterile vacutainer blood collection tubes containing EDTA as coagulant. Plasma was separated from each sample and both plasma and packed cells were stored at -20oC until analysed. Initial HIV screening was by Genscreen Ultra HIV Ag-Ab (BIORAD, France) and confirmation was by the Western blot assay using New Lav Blot 1 (BIORAD, France). The results were interpreted according to the manufacturer's instructions

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Genomic DNA was extracted from whole blood using the QiaAmp DNA Blood Mini kit (Qiagen, Maryland, USA) according to manufacturer's instruction. The env C2-V3 region of HIV-1 from the genomic DNA extract was amplified by nested PCR using primers WT1 and WT2, and KK40 and KK30 for the first and second rounds, respectively (Kanki *et al*., 1999). Amplified DNA was directly sequenced in the Genetic Analyzer 3130xl (Applied Biosystem, California, USA) in both forward and reverse directions using Sequencing kit v3.1 (Applied Biosystem, California, USA). The sequences were manually edited using the sequencing software v3.1 (Applied Biosystem, California, USA) and MEGA 5.03, and analysed for phylogenetic relationships with reference sequences from the HIV database (Los Alamos, New Mexico, USA) by ClustalW and Maximum Likelihood methods in MEGA 5.03 software based on their nucleotide sequences.

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RESULTS

The target region of approximately 350bp was successfully sequenced from 64 (75.3%) of the 85 HIV-1 infected antiretroviral therapy-naïve individuals. The 64 HIV-1 infected individuals comprised of 34 males and 30 females with a median age of 37 years (range 18-58 years). Overall, 3.13%, 53.13%, 28.13%, 14.06% and 1.56% of the HIV-1 env C2-V3 nucleotide sequences were identified as subtype A, G, CRF02_AG, CRF06_cpx, and CRF35_AD, respectively (Figure 1).

The distribution of the 64 HIV-1 env C2-V3 sequences by year of sample collection is as follows: 17 sequences in 2004; 33 sequences in 2005; and, 14 sequences in 2006. The variation in subtype distribution by year of sample collection is as shown in Figure 2.

Further phylogenetic analysis of the 34 env C2-V3 subtype G sequences showed that the subtype G viruses form five different subclusters (Figure 3). Five subtype G sequences clustered with reference sequences from Estonia, two subtype G sequences clustered with a reference sequence from Sierra Leone, 13 subtype G sequences clustered with a reference sequence from Ghana and 9 subtype G sequences clustered with a reference sequence from Nigeria. However, 5 sequences clustered on their own in the G cluster which is referred to as G'.

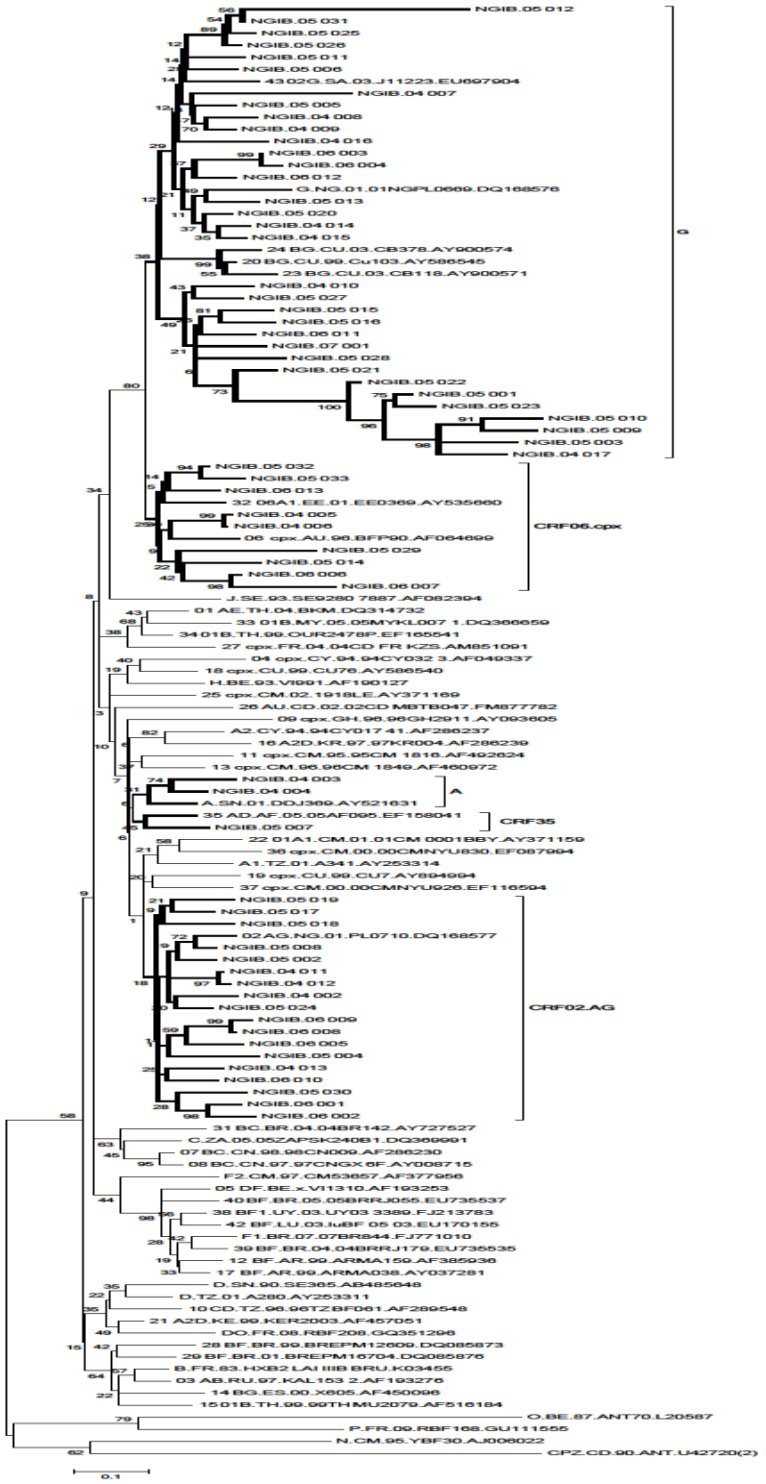

Figure 1:
Phylogenetic distribution of HIV-1 strains in Ibadan, Nigeria

DISCUSSION

Molecular epidemiologic analyses is a powerful tool that is being used for monitoring the evolution of the HIV-1 epidemic in different populations and geographic regions, especially in Africa where the HIV/AIDS pandemic is very dynamic (Hemelaar *et al*., 2006). Knowledge of circulating HIV-1 strains will help to understand HIV-1 molecular epidemiology, its pathogenic consequences and inform the design of candidate vaccines.

Results from studies on molecular analysis of HIV-1 in Nigeria have shown high dynamism and increasing complexity among strains of the virus by the identification of new variants, including circulating and unique recombinant forms (Sankale *et al*., 2007), recognition of new outbreaks and changes in established epidemics as well as the characterization of partial and full-length genomes.

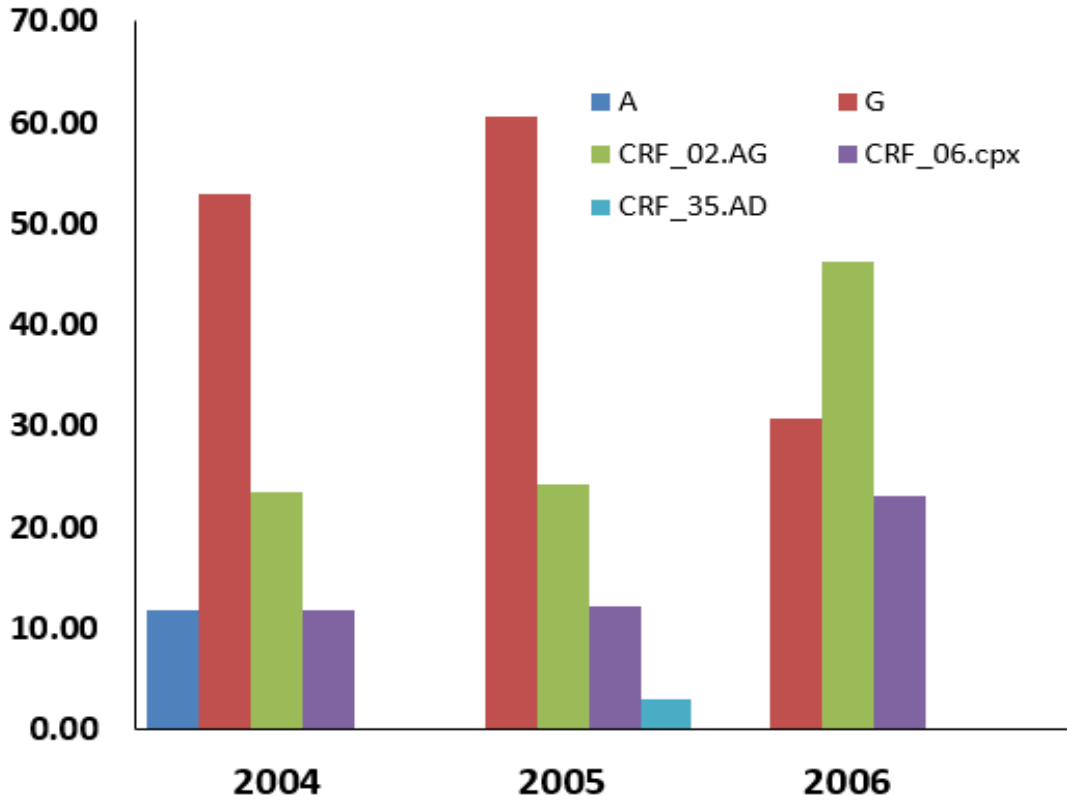

Figure 2:
HIV-1 Subtype Distribution by Year In Ibadan, Nigeria

HIV-1 genotyping results for env C2-V3 sequences were obtained for 75.3% (64/85) of the samples analyzed and it shows circulation of HIV-1 subtypes A (3.13%), G (53.13%), CRF02_AG (28.13%), CRF06_cpx (14.06%) and CRF35_AD (1.56%) among HIV-1 infected individuals in the study population. This data suggests that the level of diversity of the virus among the study population is complex, dynamic and evolving, and agrees with reports from previous molecular studies of HIV-1 diversity in Ibadan as well as other cities, states and regions in Nigeria (Agwale *et al*., 2002; Ajoge *et al*., 2011; Chaplin *et al*.; Odaibo *et al*., 2006; Ojesina *et al*., 2006; Peeters *et al*., 2000; Sankale *et al*., 2007). The differences observed between the HIV-1 subtype prevalence results of this study and that of the previous studies in Nigeria could be attributed to the differences in the circulating strains with respect to locality; the year of study; and, the type of assay used for subtype determination. Moreover, these studies clearly show on-going virus diversification. Thus, the distribution of HIV-1 genetic diversity with respect to geographic location is highly dynamic with novel genetic diversity continually being generated through mutation and recombination with travel and migration promoting the transfer of diverse viral strains within and between populations over time (Gifford *et al*., 2007), which has shown by the different molecular studies of HIV-1 in Ibadan.

Another significant finding from this study was the detection of circulation of HIV-1 CRF35_AD. This is the first report of HIV-1 CRF35_AD in Nigeria, and indeed, sub-Saharan Africa. This circulating recombinant form has previously been reported as the virus strain largely driving the HIV-1 epidemic among intravenous drug users (IDUs) in Kabul (Sanders-Buell *et al*., 2007) and Hirat (Sanders-Buell *et al*.), 2 cities in Afghanistan. This CRF has also been reported among HIV-1 infected patients in Iran (Soheilli *et al*., 2009) and Pakistan (Shah *et al*.). The presence of this CRF suggests its introduction into Nigeria from relationships with the Middle East through travel and migration since it has been established that travel and migration promote the transfer of diverse viral strains between populations, and often across large distances (Perrin, Kaiser, & Yerly, 2003; Thomson & Najera, 2005). Travellers contribute to the spread of HIV-1 genetic diversity worldwide, and in the developing world migration of rural populations and civil war are additional contributing factors (Thomson & Najera, 2005). In addition, the detection of this CRF may be an indication that there are yet other unrecognized CRFs and URFs in the country. Thus, the complexity of the HIV-1 epidemic in Ibadan, and indeed Nigeria, may be very well underrepresented.

HIV-1 subtype G was the dominant (53%) or most prevalent subtype among multiple strains of circulating HIV-1 viruses characterized in this study. which is also consistent with a report by Agwale *et al*. (Agwale *et al*., 2002). However, some other investigators have reported other subtypes of HIV-1 as the predominant subtype in circulation. In West Africa, most of the epidemic is believed to be due to subtype A and recombinant viruses including subtype A, in particular CRF02_AG (Montavon *et al*., 2000; Peeters *et al*., 1998; Sankale *et al*., 2000). Peeters *et al*. (Peeters *et al*., 2000) and Agwale *et al*. (Agwale *et al*., 2002) reported a predominance of subtype A viruses in southwestern Nigeria and subtype G viruses in Northern. A report by Chaplin *et al*. (Chaplin *et al*.) involving 4 treatment sites in Nigeria showed the predominance of HIV-1 CRF02_AG overall but with the predominant subtype varying among the different treatment sites (Chaplin *et al*, 2011).

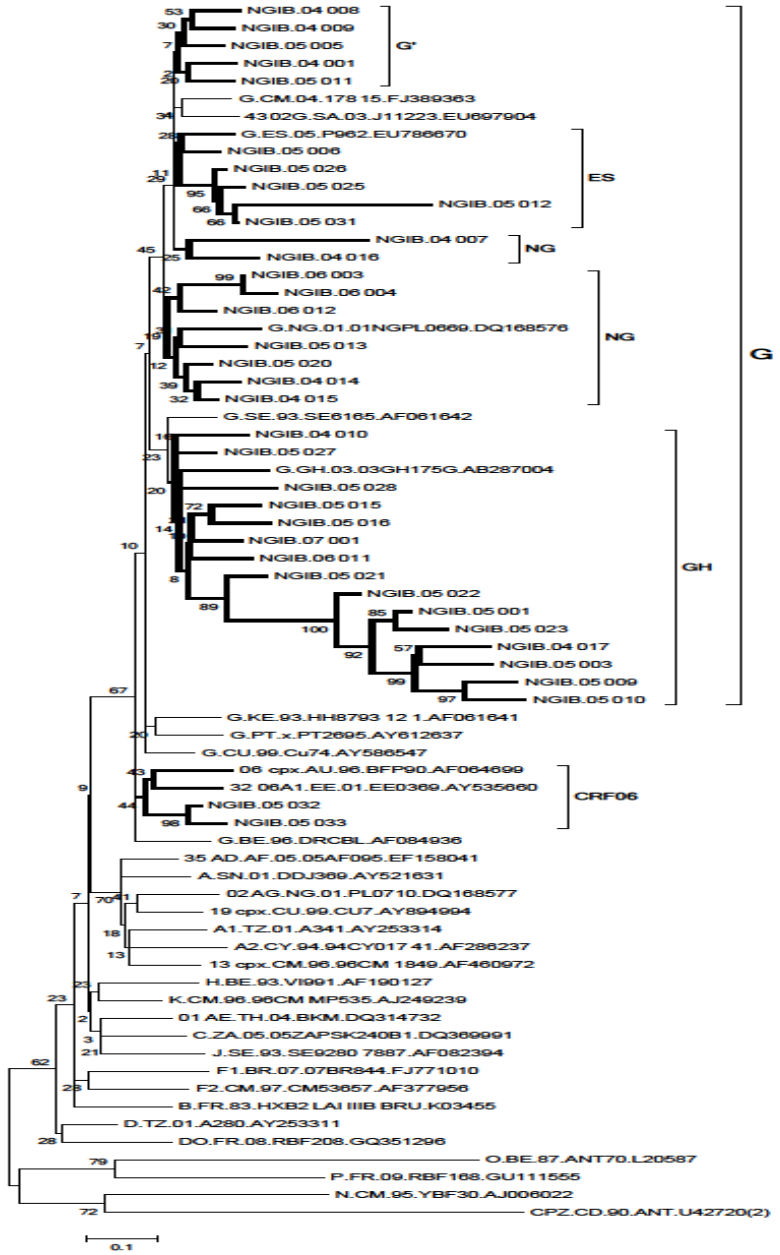

Figure 3:
Distribution of Hiv-1 Subtype G Sequences In Ibadan, Nigeria

HIV-1 CRF02AG was reported as the predominant circulating HIV-1 strain by Ajoge *et al*. (Ajoge *et al*., 2011) and Sankale *et al*. (Sankale *et al*., 2007). According to a report for HIV-1 infection in West Africa, the dominant HIV-1 subtypes are A (21%), G (35%), CRF02_AG (28%), and other recombinants (14%), most of which is CRF06_cpx, leaving the other subtypes at less than 1% each (Hemelaar *et al*., 2006). The results of this study agrees with this report and show that subtype G accounts for 53% with subtype A and its recombinant, CRF02_AG, accounting for about 31% of circulating HIV-1 strains. Consequently, HIV-1 subtypes G and A, including CRF02_AG, make up 84% of the circulating HIV-1 strains in this study. Most previous studies on HIV-1 diversity in Nigeria have shown that subtype G and subtype A, including its recombinant CRF02-_AG make up above 80% of the proportion of circulating viruses (Agwale *et al*., 2002; Ajoge *et al*., 2011; Ojesina *et al*., 2006; Sanders-Buell *et al*.; Sankale *et al*., 2007).

Among the 34 subtype G sequences, 5 (14.7%) formed a unique monophyletic subcluster. This subcluster has been referred to as G' (G prime). HIV-1 subtype G' has been reported from different molecular epidemiologic studies of HIV gp41 (Howard *et al*., 1994; Peeters *et al*., 2000), pol (Chaplin *et al*.; Ojesina *et al*., 2006), env (Ajoge *et al*., 2011) and gag (Sankale *et al*., 2007) from different parts of Nigeria (Ajoge *et al*., 2011; Chaplin *et al*.; Howard *et al*., 1994; Ojesina *et al*., 2006; Peeters *et al*., 2000; Sankale *et al*., 2007). The relationship of this subtype G' to the prototypical subtype G for future immunological and vaccine research in Nigeria, may only be accessible through full-length genome amplification.

There was variation in HIV strain and prevalence over the 3 years of sample collection from 2004 to 2006 thus, emphasizing the evolving and dynamic nature of the HIV-1 epidemic. Of the 5 HIV-1 strains found in this study, 3strains (G, CRF02_AG and CRF06_cpx) were detected from HIV-1 positive blood samples every year, although, in varying proportions. Subtype A was only detected among HIV-1infected patients in 2004 while CRF35_AD was detected among HIV infected patients in 2005. Also, while the proportion or prevalence of CRF02_AG and CRF06_cpx viruses increased from 2005 to 2006, the proportion of subtype G significantly reduced. This dynamic and evolving picture of the HIV-1 epidemic is apparent from the comparison of previous reports of studies on HIV-1 genetic diversity in Nigeria (Ajoge *et al*., 2011; Chaplin *et al*.; Howard *et al*., 1994; Ojesina *et al*., 2006; Peeters *et al*., 2000; Sankale *et al*., 2007). These studies clearly show endemicity of HIV-1 subtypes G and CRF02_AG as well as virus evolution and diversification reflected by increasing virus complexity and detection of newer strains, with increasing proportion of recombinants.

Some CRF specific characteristics were also observed among the HIV-1 viruses in this study. About 43% of the HIV-1 viruses from this study are recombinant viruses; recombinants of subtypes A and G, predominantly. This is consistent with findings that more than 20% of the current HIV-1 infections in Africa are estimated to be recombinant strains (McCutchan *et al*., 1999; van der Kuyl & Cornelissen, 2007). HIV-1 recombinants arise as a result of dual infections with HIV-1 strains, which can be individual subtypes or CRFs. Although, the possible consequences of the emergence of recombinant HIV-1 strains is not yet fully understood, the presence of this large number of recombinants may be another

indication that HIV-1 diversification is continuing in this locality. Also, if recombinants could have increased fitness over the parental strains as in vitro models suggest, and could exhibit increased pathogenicity, then future studies on HIV-1 sequence data would show increasing proportion of recombinant viruses. Moreover, multiple drug resistant (MDR) strains could recombine to produce a pan-resistant, transmittable virus (van der Kuyl & Cornelissen, 2007). In addition, if it is eventually discovered or proved that the efficacy of an HIV-1 vaccine is subtype specific, the presence of this large number of recombinants may present some challenge to vaccine development (Sankale *et al*., 2007).

The primers used in this study have been previously used in other molecular epidemiology studies in West Africa (Kanki *et al*., 1999; Sankale *et al*., 2000; Sankale *et al*., 2007). When the distribution of the subtypes of HIV-1 in this study was examined according to the demographic of the patients no association was found between HIV-1 subtype and age or gender.

In conclusion, HIV-1 genotyping results of env C2-V3 sequences were obtained for 64 (75.3%) of the 85 HIV-1 positive blood samples analysed. This study has shown for the first time, the circulation of HIV-1 CRF35_AD in Nigeria among multiple strains of HIV-1 including subtypes A, G, CRF02_AG, and CRF06_cpx among HIV-1 infected individuals. HIV-1G accounts for the majority of circulating strains and there was observed year to year variation in subtype prevalence and distribution among HIV-1 infected persons.

This study involved an approximately 350bp gene fragment from env and it is possible that strains characterized as one subtype or CRF may be recombinant viruses. Additional work, involving two or more genes or full-length sequencing, is needed to fully characterize the viruses circulating in this region and other parts of the country. At the molecular level, the HIV-1 epidemic in some cities and states of Nigeria have been previously characterized, thus, this study does complement and add value to the current understanding of the HIV-1 epidemic and the genetic variants of HIV-1 in Nigeria.

Supporting Data: *The sequences have been submitted in GenBank with Accession numbers KF437580-KF437619 and KP751963-KP751959.*

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Authors' Contributions: *Donbraye E' participated in the design of the study, performed the laboratory and sequence data analysis and wrote the first draft of the manuscript. 'Odaibo GN' participated in the design and coordination of the study, jointly developed the structure and analysis for the manuscript and revision of the manuscript. 'Olaleye DO' conceived idea of the study, participated in its design and coordination, made critical revisions and approved the final version of the manuscript. All authors read and approved the final manuscript."*

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