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Research article

Phylogeny of partial *gag*, *pol* and *env* genes show predominance of HIV-1G and CRF02_AG with emerging recombinants in south-eastern Nigeria

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ABSTRACT

Human Immunodeficiency Virus is characterized by high degree of genetic diversity with marked differences in its geographic distribution even within a country. This study was designed to identify the strains of HIV-1 circulating among infected individuals in southeastern parts of Nigeria.

Genomic DNA was extracted from blood samples of 30 HIV-1 infected individuals from Anambra, Delta and Imo states of southeastern Nigeria. Portions of the genome corresponding to entire p24 *gag*, entire *protease* and C2–V3 *env* genes were amplified by nested PCR, sequenced using Sanger's method and phylogenetically analysed.

Out of the 30 samples sequenced, 17, 28 and 14 readable sequences were obtained for *gag*, *pol* and *env* regions respectively. The most prevalent subtypes were CRF02_AG (41.2% in *gag*, 57.1% in *pol protease* and 50.0% in *env*) and G (29.4% in *gag*, 35.7% in *pol protease* and 35.7% in *env*). Other subtypes identified include A (17.7% in *gag*, 7.1% in *env*) and J (7.1% in *env*). Also 2 sequences each in *gag* (11.8%) and *pol protease* (7.1%) regions were unclassified but preliminary analysis showed they are recombinants. Furthermore, 71.4% of the isolates with sequences in the 3 regions and 26.7% of those with sequences in 2 genomic regions were recombinant forms.

CRF02_AG and subtype G are the predominant HIV-1 strains circulating among infected individuals in southeastern Nigeria. Preliminary analysis results of unclassified sequences suggest that they are new recombinants.

1. Introduction

Human Immunodeficiency Virus type 1 (HIV-1) is genetically highly diverse as a result of its high rate of replication [1, 2], mutation [3] and recombination [4, 5, 6]. Presently, there are four groups of HIV-1 including M (major), O (outlier), N (new) and P (putative). Group M is responsible for ˃90% of cases of HIV infection globally [7] and has evolved into multiple genetic forms termed subtypes, subsubtypes, circulating recombinant forms (CRFs) and unique recombinant forms (URFs). Nine subtypes which include A-D, F–H, J and K have been identified till date. CRFs arise as a result of recombination events occurring between two or more subtypes. Presently, more than 79 CRFs have been identified and more are still being identified. In addition, URFs are being reported in individuals infected with two or more HIV-1 clades in areas where multiple variants are circulating [8].

The distribution of the different variants varies by regions globally. While clade B is predominant in Europe and the Americas, the epidemic in Southern and Eastern Africa, India and Nepal is predominated by clade C [9, 10]. In West and West Central Africa, most viruses are CRF02-AG [11, 12, 13] while subtype D is generally limited to East and Central Africa, with sporadic cases observed in Southern and Western Africa [14, 15, 16]. Pure clade E has never been isolated but rather appears as an A/E mosaic which has been detected in Thailand, the Philippines, China and Central Africa [14, 15]. Subtype F has been reported in Central Africa [17, 18], South America [19] and Eastern Europe [20, 21] while G and A/G recombinants have been observed in Western and Eastern Africa as well as in central Europe [15]. Subtypes H and J were described in Central Africa [22, 23, 24]) and in Angola [25, 26]). Clade K has been identified in the Democratic Republic of Congo and Cameroon [27].

In Nigeria, the complex nature of the circulating strains has been demonstrated [28] with multiple subtypes and CRFs of HIV-1 and HIV-2 already reported [29, 30, 31, 32, 33, 34]. Most of these have been based on isolates from the south-western and northern regions of the country. With attention gradually shifting to development of candidate vaccine, its success in Nigeria will largely depend on adequate knowledge of all the circulating strains of the virus from the different regions of the

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country. In this study we determined the HIV-1 strains circulating in south-eastern Nigeria.

2. Materials and methods

2.1. Study population

The study population consisted of 30 consenting HIV-1 infected individuals assessing HIV clinics at General Hospital Awo-Omamma, Imo state, State Hospital Asaba, Delta state and St Joseph's Catholic Hospital Adazi Anambra state between February and May 2012. They included 11 males and 19 females with mean age of 39.6 years (range: 3–70 years). Ethical approval UI/EC/11/0178 was obtained for this study from University of Ibadan/UCH ethical review board and the study conformed to Ethics of Human subjects use in research following the Helsinki Declaration. Informed consent was obtained from adult participants, while parental assent was obtained from young persons under 18 years of age.

2.2. Sample collection, preparation and storage

Blood sample was collected from each patient by venepuncture into Ethylene Diamine Tetra Acetic Acid (EDTA) bottle and properly labelled with date of collection and laboratory identification number. Plasma was separated from each sample by centrifugation at 3000 rpm for 5 min and aspirated into sterile cryovials. Both the plasma and packed cells were stored at -20 °C until analysed.

2.3. DNA extraction and precipitation

Genomic DNA was extracted from the packed blood cells using modified phenol-chloroform extraction procedure followed by ethanol precipitation [35]. Five hundred microliters (500μl) of packed blood cells was dispensed into Eppendorf tube and equal volume of equilibrated phenol/chloroform (50:50) was added. The tube was vortexed vigorously and spun in a centrifuge at top speed (16,000 rpm/25,000 rcf) for 3 min to separate the phases. The aqueous phase was aspirated into a new Eppendorf tube and equal volume of chloroform added. The tube was then vortexed vigorously and spun as above to remove any traces of phenol. The aqueous phase was aspirated into a new Eppendorf tube carefully. This step of addition of chloroform to remove traces of phenol was repeated until no visible protein interface between the chloroform and the aqueous phase was seen. The aqueous phase was aspirated into a new Eppendorf tube carefully. Fifty microliters (50μl) of 3M sodium acetate and 1 ml of 100% ethanol was added into the tube. The tube was vortexed and frozen at -80 °C for 40 min. The tube was spun at full speed in a centrifuge for 30 min at 4 °C and the supernatant decanted. Three hundred microliters (300μl) of 70% ethanol was added to the DNA pellet, vortexed and spun for 5 min at 4 °C. The supernatant was decanted and the pellet was air dried at 37 °C for approximately 10 min. The DNA was re-suspended by adding 50μl of 1X TE buffer. The tube was then vortexed and centrifuged for 10 s to pool the sample and then stored at -20 °C until used. The extraction was done inside level 3 biosafety cabinet.

2.4. PCR amplification

Nested PCR was used for the amplification of regions of HIV-1 *gag*, *pol* and *env* genes corresponding to entire p24 *gag* region, the entire 297 bp of protease (PR) region and C2–V3 *env* domain. Details of the primers used are shown in Table 1. The thermal-time profile for the amplification of p24 *gag*, pol *protease* and *env* C2–V3 regions are as previously described by Ajoge *et al.* [32], Ojesina *et al.* [36] and Kanki *et al.* [37] respectively. The reactions were performed in a thermal cycler (Applied Biosystems GeneAmp PCR System 9700®). PCR reactions were carried out in a 50μl volume reaction mixture containing 10 μl of DNA and 10 μl of amplicon for first and second round reactions respectively, 10 μl (1X) of PCR mix (Jena Bioscience, Germany), 2 μl (0.4pmol) each of the primers and 26 μl of nuclease-free water. Electrophoresis of the amplicons was run using 1.5% agarose gels stained by ethidium bromide at 120 V and a current of 500 mA for 60 min. The bands were visualized using Bio Rad gel documentation system.

2.5. Sequencing

Only samples with positive PCR products were sequenced for this study. PCR products were purified using WIZARD Purification Kit Protocol (Promega) and subjected to direct sequencing of both sense and antisense strands using Big Dye Terminator Cycle Sequencing Ready Reaction kit v3.1 (Applied Biosystems, Foster City, CA, USA) with the second round primers and then analysed in the Genetic Analyzer 3130xl (Applied Biosystems, California, USA). All nucleotide sequences obtained in this study have been deposited at the GenBank and assigned the accession numbers MF458121-MF458137 for *gag* sequences, MF458138-MF458165 for *protease* sequences and MF581058-MF581071 for *env* sequences.

2.6. Phylogenetic analysis

Sequences were manually edited using combination of CLC BIO (QIAGEN®, USA), MEGA 6.06 [38] and sequence scanner v1.0 (Applied Biosystems, USA) software. The sequences were aligned by ClustalW with reference sequences from HIV Sequence Database at Los Alamos (www.hiv.lanl.gov). Phylogenetic inferences were performed by the neighbour-joining method with 1,000 bootstrap replicates under Kimura's two-parameter correction using MEGA 6.06. The evolutionary distances were computed using the Maximum Composite Likelihood

Table 1. Details of the primers used for the amplification of regions of *gag*, *pol* and *env* genes.

Region	Name of primer	Primer Sequence (5′–3′)	HXB2 Position	PCR	Orientation	Size of Product (bp)	Reference
P24 *gag*	G00	GACTAGCGGAGGCTAGAAG	764–782	1st round	Forward	1518	[32]
	G01	AGGGGTCGTTGCCAAAGA	2264–2281	1st round	Reverse		
	G60del3′G	CAGCCAAAATTACCCTATAGTGCA	1173–1197	2nd round	Forward	717	
	G25	ATTGCTTCAGCCAAAACTCTTGC	1867–1889	2nd round	Reverse		
Protease	OJ1	AAATGATGACAGCATGTCAGGGAG	1823–1846	1st round	Forward	2375	[36]
	OJ2	TATCTACTTGTTCATTTCCTCCAAT	4173–4197	1st round	Reverse		
	OJ3	AGACAGGCTAATTTTTTAGGGA	2074–2095	2nd round	Forward	524	
	OJ4	CATTCCTGGCTTTAATTTTACTGG	2574–2597	2nd round	Reverse		
Env C2–V3	WT1	GCTGGTTTTGCGATTCTAAAGTGTA	6884–6908	1st round	Forward	494	[37]
	WT2	CAATAGAAAAATTCCCCTCCACAAT	7353–7377	1st round	Reverse		
	KK40	ACAGTACAATGTACACATGG	6954–6973	2nd round	Forward	384	
	KK30	AATTTCTGGGTCCCCTCCTG	7318–7337	2nd round	Reverse		

method and are in the units of the number of base substitutions per site [39].

3. Results

3.1. HIV-1 subtypes

Out of the 30 samples analysed, 17 *gag*, 28 *pol* and 14 *env* sequences were obtained. Phylogenetic analysis showed that out of the 17 *gag* sequences, 3 (17.7%), 5 (29.4%) and 7 (41.2%) were HIV-1A, HIV-1G and CRF02_AG respectively while 2 (11.8%) did not cluster with any of the known subtypes or CRFs. Blast results of these two sequences, NG_AN.12_04 and NG_AN.12_07 from the Los Alamos HIV-1 sequence database showed that the isolates had closest similarity to HIV-1 CRF02_AG and are hereby referred to as unclassified 02AG (U^{02AG}). For the *pol* sequences, 10 (35.7%) and 16 (57.1%) were HIV-1G and CRF02_AG respectively while 2 (7.1%) sequences did not cluster with any of the known subtypes or CRFs. Blast results of these two sequences, NG_DE.12_09 and NG_IM.12_06 from the Los Alamos HIV-1 sequence database also showed that the isolates had closest similarity to HIV-1 subtype G and are hereby referred to as unclassified subtype G (U^{G}). Analysis of *env* sequences showed that 5 (35.7%) were HIV-1G, 7 (50.0%) CRF02_AG, 1 (7.1%) HIV-1A and 1 (7.1%) HIV-1J. Neighbour joining (NJ) tree analysis of *gag*, *pol* and *env* sequences are shown in Figures 1, 2, and 3 respectively.

3.2. Genetic diversity

Seven (23.3%) of the 30 isolates were sequenced in the 3 fragments of *gag*, *pol* and *env* genes, 15 (50.0%) were sequenced in two of the three fragments while only one fragment was sequenced in 8 (26.7%) (Table 2). Further analysis showed that only 2 (28.6%) of the 7 isolates that were sequenced in the 3 fragments had subtype concordance in all the regions while 5 (71.4%) showed discordant subtypes/forms. Out of the 15 isolates that were sequenced in two fragments, 11 (73.3%) were subtype concordant in both regions while 4 (26.7%) showed discordant subtypes/forms (Figure 4).

4. Discussion

This study shows the circulation of HIV-1 subtypes A, G, CRF02_AG, and J in different proportions among HIV-1 infected individuals in Southeastern Nigeria. The predominance of CRF02_AG; 41.2% in *gag* region, 57.1% in *pol protease* region and 50% in *env* C2–V3 region and subtype G; 29.4% in *gag* region, 35.7% in *pol protease* region and 35.7% in *env* C2–V3 region in this study is consistent with previous reports on isolates from other parts of the country. In their separate studies, Odaibo *et al.* [40], Ojesina *et al.* [36], Sankale *et al.* [28], Fayemiwo *et al.* [33] and Donbraye *et al.* [34] reported predominance of CRF02_AG and subtype G in circulation in Ibadan, Oyo state, south-western Nigeria. Similarly, molecular studies conducted in Jos, north central Nigeria at different times also reported the predominance of subtypes G and CRF02_AG [32, 41]. Also in a more widespread study covering Lagos and Ibadan (South West), Jos (North Central) and Maiduguri (North East) Nigeria by Chaplin *et al.* [42] which evaluated RT and PR genes of 338 samples, 45.0% of CRF02_AG, 37.9% of subtype G, 4.4% of CRF06_cpx and 3.6% of subtype A were reported while others accounted for the remaining 9.2%. These studies all show the predominance of HIV-1 CRF02_AG in Nigeria which is believed to be largely driving the epidemic in the country. Our study also shows the circulation of HIV-1 subtype A at 17.7% and 7.1% based on the regions of HIV-1 *gag* and *env* C2–V3 genes respectively. Although this subtype accounted for relatively small proportion in this study, it has earlier been reported by Peeters and Sharp [13] as the major circulating subtype accounting for 61.3% in a study that included isolates from four states of the country. Similarly, a national molecular epidemiologic survey of HIV-1 strains that included samples from 34 of the 36 states of Nigeria reported a high percentage (44.8%) of HIV-1 subtype A based on *env* gp41 gene [43]. It is interesting to know from these reports that the studies conducted in early year 2000 showed higher proportion of HIV-1 subtype A in circulation in different regions of Nigeria. More recent studies including ours however, are increasingly showing predominance of CRF02_AG and subtype G in different regions of the country. Previous researchers have reported that 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 [44]. The prototype CRF02_AG (IbNg) was initially characterised as subtype A based on partial genome sequencing [45, 46].

Another significant finding from this study is the identification of HIV-1 *env* C2–V3 subtype J isolate. As far as it can be ascertained, this is the second report of this subtype in Nigeria after it was first reported in 2002 by Agwale and colleagues [43]. Detection of HIV-1 subtype J in the south east may be an indication that the virus strain is spreading to other parts of the country. HIV-1 subtype J has been reported in Democratic Republic of Congo, Angola, Botswana, Cameroon, Central African Republic, Gabon, Senegal, Uganda and Zambia and some European countries like Sweden, Belgium, Spain, France, Portugal, Germany and Italy as well as United States of America and Cuba (Los Alamos HIV Database). However, the isolate in this study closely clustered with sequence from Cameroon which shares common border with Nigeria suggesting that the strain may have been introduced into the country by trans-border travels between these two countries. Travelers 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 [47, 48]. Interestingly, this isolate, NG_IM.12_09 grouped with subtype J in the *env* region same as the one earlier reported by Agwale and colleagues. In the *gag* region however, it grouped with CRF02_AG while sequence was not available for classification in the *pol* region. This recombinant virus may represent a new recombinant form whose true mosaic structure can only be ascertained by full-length genome sequence analysis.

Also of importance in this study is the identification of two isolates, NG_AN.12_04 and NG_AN.12_07 (U^{02AG}s), which did not cluster with any known subtype or CRF in the *gag* p24 phylogenetic tree. NG_AN.12_04 was classified as CRF02_AG in both *pol protease* and *env* C2–V3 regions. NG_AN.12_07 was also classified as CRF02_AG in the *pol protease* region while sequence was not available for classification in the *env* C2–V3 region. Similarly two isolates, NG_DE.12_09 and NG_IM.12_06 (U^{G}s) also failed to cluster with any known subtype or CRF in the *pol protease* phylogenetic tree. In the *gag* and *env* C2–V3 trees, isolate NG_IM.12_06 grouped with subtype G while there was no available sequences for NG_DE.12_09 for classification in the *gag* and *env* regions. Inability of these sequences to cluster with any of the subtypes could be as a result of one or more of the following: (1) insufficient phylogenetic information within the gene fragment, (2) presence of recombinant breakpoint within the sequence that could not be detected in the gene fragment or (3) the gene regions belonged to some previously unidentified virus. Indeed, these sequences may represent new and emerging CRFs or unique recombinant forms (URFs) in the study location. This further reflects the complexity of genetic diversity of HIV-1 strains circulating in Nigeria as earlier reported [28].

Another striking observation in this study is the level of discordant subtypes recorded among the isolates in which more than one genomic region were genotyped. Of the 7 samples sequenced in the three regions of *gag*, *pol* and *env* genes, 3 (42.9%) had discordant subtypes in the three genomic regions. In the 15 samples where two genomic regions were sequenced, 3 (20%) had discordant subtypes while the rest had concordant subtypes. The frequent non-concordance among the PCR results for the *gag*, *pol* and *env* gene regions might be due to marked genetic heterogeneity of the virus in these gene regions. This also demonstrates the complex diversity of the strains of HIV-1 circulating in south-eastern part of Nigeria and hence the need to sequence the complete genome of these

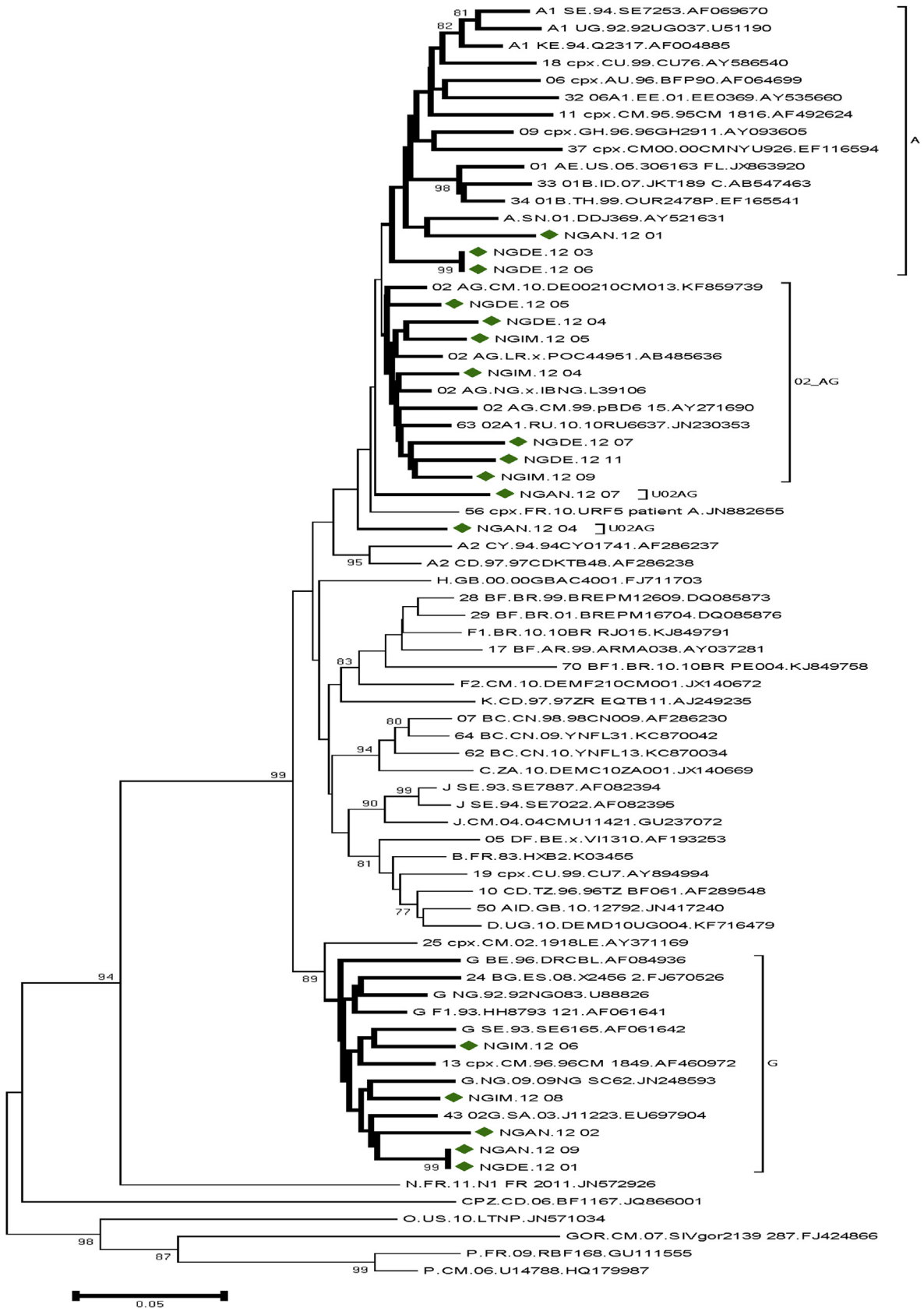

Figure 1. Phylogenetic tree of study *gag* sequences and reference sequences from Los Alamos database (www.hiv.lanl.gov). Only bootstrap values (1000 replicates) ›70% are shown. The study sequences are indicated by "◆" symbol. It indicates isolates from eastern Nigeria.

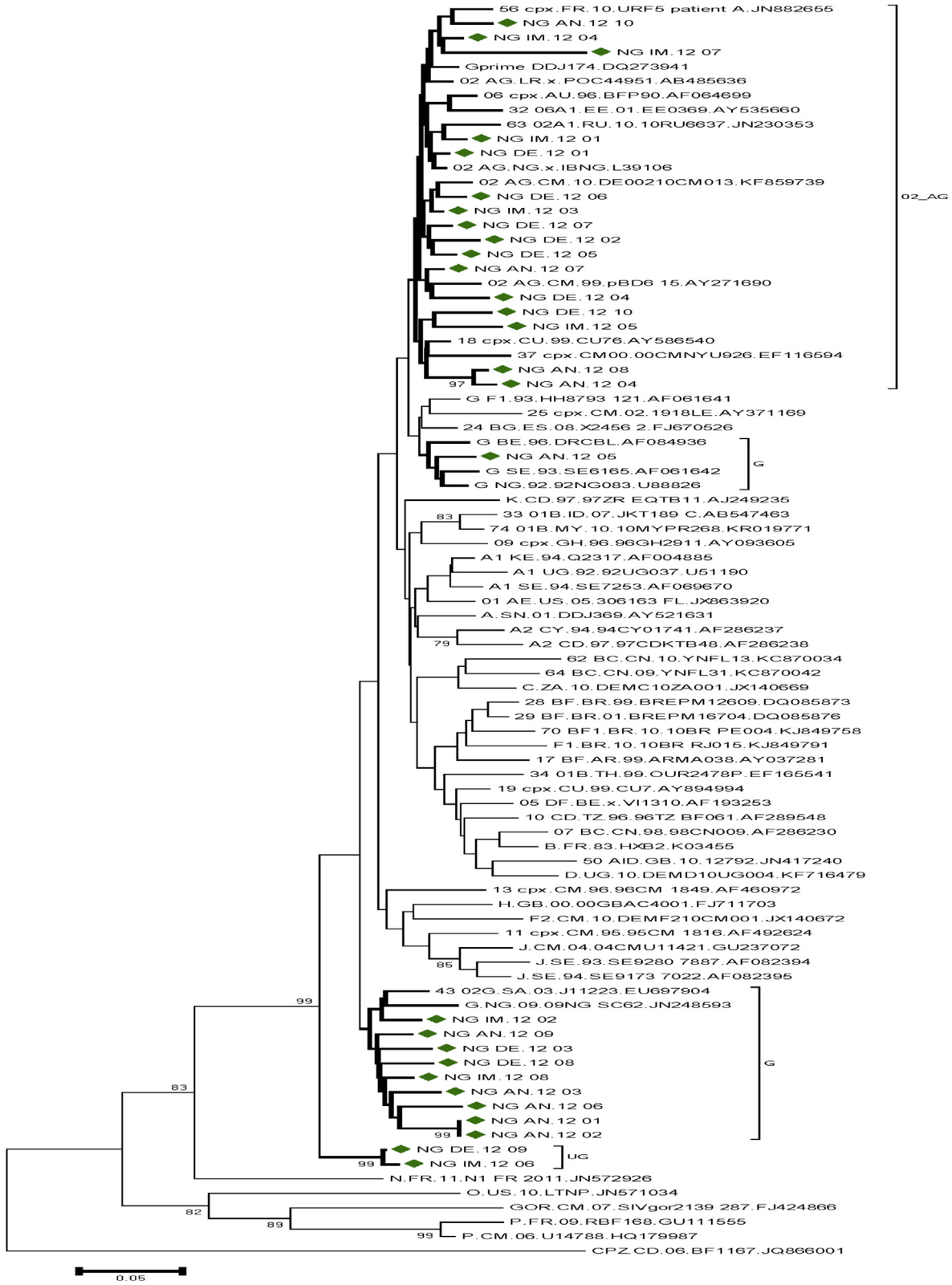

Figure 2. Phylogenetic tree of study *pol* sequences and reference sequences from Los Alamos database (www.hiv.lanl.gov). Only bootstrap values (1000 replicates) >70% are shown. The study sequences are indicated by "◆" symbol. It indicates isolates from eastern Nigeria.

isolates:- recombinant strains have been observed mostly in areas where multiple subtypes co-circulate [49]. Mosaic viruses may have biological advantages over the parental strains as the "hybrid virus" which emerged as a result of recombination events between two or more variants could acquire some advantageous traits from the parental strains. This could manifest in the modification of cell tropism, enhanced co-receptor usage, changes in growth kinetics, and improved viral fitness. The clinical implication of this changing landscape of HIV strains in the region can be quite enormous. The sensitivity of diagnostic kits can be affected as well as reduction in the susceptibility of the isolates to the existing

Figure 3. Phylogenetic tree of study V3 loop sequences and reference sequences from Los Alamos database (www.hiv.lanl.gov). Only bootstrap values (1000 replicates) ˃70% are shown. The study sequences are indicated by "◆" symbol. It indicates isolates from eastern Nigeria.

Table 2. Frequency of occurrence of HIV-1 subtypes and forms among the patients.

GAG/POL/ENV Subtype/CRF	Number of samples (%)	Total (%)
A/G/A	1 (3.3)	7 (23.3)
U^{02AG}/CRF02_AG/CRF02_AG	1 (3.3)	
G/G/G	1 (3.3)	
G/CRF02_AG/CRF02_AG	1 (3.3)	
CRF02_AG/CRF02_AG/CRF02_AG	1 (3.3)	
CRF02_AG/CRF02_AG/G	1 (3.3)	
$G/U^{G}/G$	1 (3.3)	
G/G/#	2 (6.7)	15 (50.0)
#/G/G	2 (6.7)	
U^{02AG}/CRF02_AG/#	1 (3.3)	
#/CRF02_AG/CRF02_AG	4 (13.4)	
A/G/#	1 (3.3)	
CRF02_AG/CRF02_AG/#	3 (10.0)	
A/CRF02_AG/#	1 (3.3)	
CRF02_AG/#/J	1 (3.3)	
#/G/#	3 (10.0)	8 (26.7)
$\#/U^{G}/\#$	1 (3.3)	
#/CRF02_AG/#	3 (10.0)	
CRF02_AG/#/#	1 (3.3)	

Key: # = Sequence not available, U^{02AG} = unclassified CRF02_AG, U^{G} = unclassified subtype G.

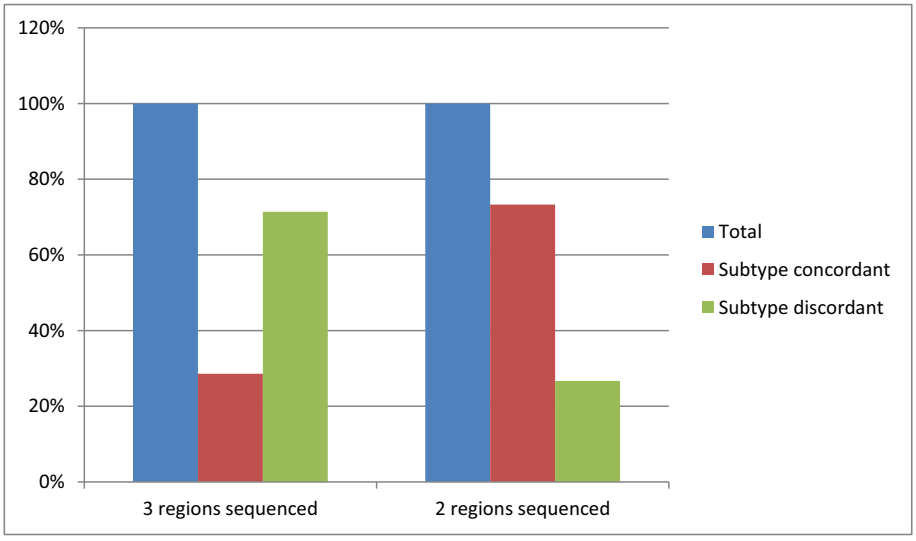

Figure 4. Percentage concordant and discordant Subtypes/CRFs among the HIV-1 infected patients.

antiretroviral drugs (ARDs) [50, 51]. This might result in the failure of existing diagnostic kits to detect certain strains of the circulating virus as well as poor prognosis of patients on the existing ARDs. This ugly scenario therefore is a cause for concern and calls for constant monitoring for the likely emergence of new/novel inter-subtype recombinant or even secondary recombinant strains as the epidemic progresses. The limitation of this study however is the small sample size analysed. The 30 samples analysed are samples in which the three genomic regions were successfully amplified out of which readable sequences were obtained for 17, 28 and 14 in the *gag*, *pol* and *env* genomic regions respectively. A larger sample size would have shown a more accurate picture of the situation. In conclusion, the predominant HIV-1 subtypes circulating in Eastern parts of Nigeria are CRF02_AG and G. Identification of mosaic strains including unclassifiable sequences highlights the complexity of HIV epidemic in the region. The unclassifiable sequences in this study may represent new/emerging strains whose true identity can only be ascertained by more elaborate study with larger sample size and full-length genome sequence analysis. The extensive variability of HIV-1 strains in the region will undoubtedly impact negatively on the development of candidate vaccine.

Declarations

Author contribution statement

Augustine O. Udeze: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed

reagents, materials, analysis tools or data; Wrote the paperdraft of the manuscript and approved the final version.

David O. Olaleye, Georgina N. Odaibo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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