Original Research Article

Co receptor Usage of Human Immunodeficiency Virus Type 1 Strains among Individuals Presenting for HIV Counselling and Testing in Ibadan, Nigeria

ABSTRACT

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Aims To evaluate HIV-1 Coreceptor usage in Nigeria.

Study design: Cross-sectional study. Place and Duration of Study: Department of Virology, College of Medicine, University

College Hospital, Ibadan from January 2004 to December 2006. **Methodology:** Genomic DNA was extracted from blood samples of 85 (42 males, 43 females; age range 18-58 years) consenting HIV-1 infected antiretroviral therapy-naïve individuals presenting at a voluntary counseling and testing centre. The *env* C2-V3 region of HIV-1 proviral DNA was amplified by nested PCR, successfully sequenced, manually edited and evolutionary relationships determined by maximum likelihood using MEGA 5.03^R from 64 of the blood samples of the HIV-1 infected patients. HIV-1 coreceptor usage was predicted based on genotypic analysis of HIV-1 *env* V3 loop sequences.

Results: Phylogenetic analysis showed HIV-1 subtypes A, G, CRF02_AG, CRF06_cpx, and CRF35_AD among the study participants The V3 loop region of the viruses had amino acid sequence conservation in the base positions 1-8 and 26-35 and tip regions and sequence variability, including mutations and deletions at positions 9-25. Most (76.6%) of the sequences had the GPGQ crown motif while the GPGQ/L/R/K substitution was observed in 18.8%. The number of N-linked glycosylation sites ranged from 0 to 4 per *env* C2-V3 amino acid sequence with only 37.5% of the sequences having all 4 N-linked glycosylation sites. Predicted frequencies for CCR5 and CXCR4 genotypes were 31.2% and 68.8%, respectively, while 10% of the CCR5-tropic viruses showed Maraviroc-associated resistant mutations.

Conclusion: CXCR4-tropic viruses predominate among the studied population irrespective HIV-1 subtype and <u>and it is associated with multiple amino acid deletions and mutations in V</u> and the loss of one or more N-linked glycosylation sites. This data suggest the need for furth studies involving <u>a</u> larger sample size prior to introduction of coreceptor inhibitors like Maravird for management of HIV infection in Nigeria.a.

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Keywords: Coreceptor usage, HIV-1 strains, CCR5, CXCR4, C2-V3 region, V3 loop

- 13 14 **1. INTRODUCTION**
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Early in infection, HIV-1 generally uses the CCR5 chemokine coreceptor, along with CD4, for 16 cellular entry [1]. In many HIV-1 infected individuals, viral genotypic changes arise that allow 17 18 the virus to use CXCR4, either in addition to CCR5 or alone, as an entry receptor [2] and this 19 change is associated with accelerated CD4 decline and more rapid progression of HIV-1 20 disease[3]. Based on chemokine coreceptor usage, HIV-1 viruses can be classified as CCR5-tropic, CXCR4-tropic or dual-tropic (R5X4) viruses [4]. The V3 loop of gp120 largely 21 determines coreceptor usage and the development of CXCR4 viruses is gradual and 22 23 involves the accumulation of multiple amino acid changes in the V3 loop [5, 6].

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25 With the introduction of HIV-1 coreceptor inhibitors as components of antiretroviral therapy 26 [7], it is increasingly imperative to screen HIV positive individuals before they commence 27 therapy with coreceptor inhibitors like Maraviroc. Maraviroc is an HIV-1 coreceptor 28 antagonist that has shown good efficacy and tolerability in treatment-naive and treatment-29 experienced patients harboring CCR5-tropic virus [8]. The use of Maraviroc in treatment 30 simplification in patients requires analysis of HIV-1 DNA.

31 However, there is little information regarding bioinformatics prediction or analysis of HIV-1 coreceptor usage in Nigeria with the only reported data by Ajoge and colleagues [9], 32 yYet this information is important for drug design and clinical management of patients before 33 34 coreceptor inhibitors are used for treatment. There is also growing evidence of 35 increasing viral resistance (primary and secondary - fuelled by both virus mutation and non-adherence issues) to commonly used anti-retroviral drugs [10-12] available 36 37 in the country and there remains a possibility that CCR5-inhibitors like Maraviroc, 38 can be used for HIV-1 antiretroviral therapy[11]. This study was therefore carried out to 39 evaluate virus coreceptor usage by bioinformatics method based on the V3 loop sequences 40 of HIV-1 from HIV-1 infected antiretroviral therapy-naïve individuals.

42 2. METHODOLOGY

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44 2.1 Study location

This study was carried out in the Department of Virology, College of Medicine, University College Hospital, Ibadan. The department offers HIV counseling and testing services as well as monitoring of Anti-Retroviral Therapy (ART) response in HIV infected patients. The department is also the Center for the WHO National Polio laboratory, HIV reference laboratory and WHO Collaborating Center for Influenza surveillance. Patients enrolled for the study were mostly from Ibadan and other locations in the southwestern region of Nigeria.

52 2.2 Study population

The study population consisted of 85 consenting HIV-1 infected anti-retroviral therapy-naïve individuals. They included 42 males and 43 females with median age of 37 years (range 18-58 years) who presented at the HIV counseling and testing centre in the Department of Virology, College of Medicine, 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.

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61 2.3 Sample collection, preparation and storage

Blood sample was collected by venepuncture from each patient into sterile vacutainer blood collection tubes containing EDTA (ethylene diamine tetra acetic acid) as coagulant. Each specimen was labelled with the date of collection and laboratory identification number. Plasma was separated from each sample and both plasma and packed cells were stored at -20°C until analysed. Only samples that were positive by Western blot for HIV-1 antibodies were analysed further for the study.

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69 2.4 Laboratory analysis

70 2.4.1 Serology

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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75 2.4.2 Molecular analysis

76 Genomic DNA was extracted from whole blood using the QiaAmp DNA Blood Mini kit 77 (Qiagen, Maryland, USA) according to manufacturer's instruction. The env C2-V3 region of 78 HIV-1 from the genomic DNA extract was amplified by nested PCR method using primers 79 WT1 and WT2, and KK40 and KK30 for the first and second rounds, respectively [13]. 80 Amplified DNA was directly sequenced with the second round primers in the Genetic Analyzer 3130xl (Applied Biosystems, California, USA) in both forward and reverse 81 82 directions using Sequencing kit v3.1 (Applied Biosystems, California, USA). The sequences 83 were manually edited using the sequencing software v3.1 (Applied Biosystem, California, USA) and MEGA 5.03, and analysed for phylogenetic relationships with reference 84 85 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 86 87 (approximately 350bp) and translated amino acid sequences [14].

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89 2.4.3 Virus phenotype determination

The virus coreceptor usage was predicted by combined criteria using a genotypic rule based on one of the following criteria for predicting CXCR4 (R4) coreceptor usage (i) Arginine (R) or Lysine (K) at position +11 of V3 and/K at position +25, (ii) R at position +25 of V3 and a net charge \geq +5, or (iii) a net charge of \geq +6 or (iv) bioinformatic analysis by comparison between Cons B and query V3 loop sequence in Geno2Pheno (10% FPR) which uses the presence of X4 specific mutations in the V3 loop as a predictor of X4 phenotype [15-19].

97 3. RESULTS AND DISCUSSION

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99 3.1 HIV-1 subtype

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, 3.1%, 53.1%, 28.1%, 14.1% and 1.6% 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].


109 110 FIGURE 1: PHYLOGENETIC DISTRIBUTION OF HIV-1 STRAINS AMONG HIV-1 POSITIVE INDIVIDUALS

112 3.2 V3 loop sequence analysis

113 The sequence analysis of the V3 loop showed total conservation for 3 (P16, G17, and A33) out of the 35 amino acid residues (8.6% of conservation). P16 and G17 are motifs within the 114 GPGQ motif of the V3 tip and A33 is in the base. The positions in which variation did not 115 exceed 10% were concentrated in the base (R3-4.7%, P4-7.8%, I30-4.7%, R31-7.8%), the 116 117 tip (G15-4.7%) and the stem (N6-3.1%, I26-6.3%). The most variable position was the 25th, 118 which was the determinant for the 11/25 rule used for predicting HIV-1 coreceptor usage. The variation in the Cysteine residues limiting the V3 loop was 3.1% for C1 and 1.6% for 119 120 C35 [Figure 2]. 121

122 The conserved GPGQ crown motif was the most common sequence observed at the V3 tip 123 occurring in 76.6% (49) of the translated HIV-1 V3 loop amino acid sequences. There was 124 substitution from GPGQ to other V3 crown motifs like GPGL (8), GPGR (3), GPGK (1),

125 APGQ (2) and RPGQ (1), as shown in figure 2.

		1	2	3	4	5	6	7	8	5	9	10	1	11	2 1	3 1	• •	15	16	17	18	19	20	21	22	23	3 2	4 3	25	26	27	28	29	30	31	32	33	34	35	Subtype	Net Charge	Geno2Pheno (1	0%)	Coreceptor
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NGIB.06.014		С	Т	R	P	S	Ν	Ν	T	-	-	R	K	Y	N	1		G	P	G	K	A	F	Y	Т	Т	C	3	E	I	I	G	D	I	R	Q	A	Н	С	G	3	X4		X4
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129 Most potential N-linked glycosylation sites were conserved in the immediate vicinity of and 130 within the V3 loop of the HIV-1 sequences analyzed. The NxS/Ty sequon for the potential N-131 glycosylation site centered at the 5'-Cys V3 loop residue was conserved in 46 (71.9%) of the 132 HIV-1 C2-V3 amino acid sequences with 41 (89.1%) presenting with the NCT (Asparagine-Cysteine-Threonine) sequon [Figure 2]. Potential N-linked glycosylation sites located six 133 134 amino acids downstream from the 5' cysteine residue in the V3 loop was conserved in 61 (95.3%) of HIV-1 infected individuals with 54 (88.5%) presenting with the NNT (Asparagine-135 136 Asparagine-Threonine) sequon.

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Interestingly, one individual had no potential N-linked glycosylation site in the HIV-1 C2-V3
 amino acid sequence analyzed. This sequence (NGIB.04_007) is a subtype G HIV-1 virus
 having the GPGQ V3 tip motif with a predicted X4 phenotype.

142 3.3 Prediction of Coreceptor usage

Using the 4 combined criteria, 68.8% (44) of V3 loop amino acid sequences had predicted
X4 virus phenotype while the remainder (31.2%) were CCR5-tropic. Of these 44 V3 loop
amino acid sequences with CXCR4 tropism, 7 V3 loops were classified as X4 phenotype
based wholly on the 11/25 rule while the remaining 37 were predicted based on a
combination of the other 3 criteria.

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149 On further analysis of the V3 loop amino acid sequences that met each criterion, 16 of the 150 V3 loop amino acid sequences had Lysine residue at position +11 while only one had 151 Arginine residue at position +11. The only V3 loop sequence with the Arginine residue at position +11 also had a lysine residue at position +25. One V3 loop amino acid sequence 152 had a net charge of +6. Bioinformatics analysis with Geno2Pheno at 10% false positive rate 153 (FPR) predicted 38 (including some V3 loop sequences that met the other criteria) as X4 154 155 viruses. All V3 loop sequences with 33 and 31 amino acid residues, and those not limited by 156 the Cysteine residues, were predicted as X4 virus phenotype. In addition, HIV-1 subtypes G 157 and CRF02_AG had a higher prevalence of CXCR4 virus phenotypes compared to HIV-1

158 CRF06_cpx in the study population [Table 1]

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160 TABLE 1. FREQUENCY OF CORECEPTOR USAGE AMONG HIV-1 SUBTYPES

	Subtype A	Subtype G	CRF02_AG	CRF06_cpx	TOTAL
Subtype	(%)	(%)	(%)	(%)	(%)
Total	2	34	18	9	63
X4 Phenotype	1(50.0)	25(73.5)	14(77.8)	4(44.4)	44(68.8)
R5 Phenotypy	1(50.0)	9(26.5)	4(22.2)	5(55.6)	20(31.2)

161

162 DISCUSSION.

163

164 The increasing interest in HIV-1 tropism is related to the introduction of CCR5 inhibitors as 165 components of antiretroviral therapy [7]. Although, the "gold standard" for characterization of 166 HIV-1 tropism is a recombinant virus phenotypic entry assay, genotypic methods based on 167 the V3 amino acid sequence analysis and characteristics have been successful with different 168 HIV-1 subtypes using sequence-based criteria derived from HIV-1 subtype B [20-169 22].Previous authors have correlated the use of sequence based criteria derived from HIV-1 170 subtype B for analysis of non-B subtypes and found it appropriate[6, 21]

Prediction of HIV-1 coreceptor usage by genotypic methods are easier to do, more cost effective and have been found to be useful as screening strategy in medical practice [23, 24]. Moreover, the anti-CCR5 inhibitor, Maraviroc, can be taken with or without food, and does not require refrigeration and thus, can be very suitable for resource-limited settings like sub-Saharan Africa.

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177 The presence of CXCR4-tropic HIV-1 isolates in HIV-1 infected individuals is associated with 178 a rapid decline of CD4+ T cells, rapid disease progression, and reduced survival time after 179 AIDS diagnosis [25]. Various studies on motif analysis of the V3 region of *env* have shown 180 certain characteristics that are strongly associated with CXCR4 usage and they include: the 181 presence and accumulation of CXCR4-associated mutations; insertions and deletions in V3, 182 the presence of basic amino acids at V3 positions 11 and/or 25, the loss of a glycosylation 183 site and an increased positive amino acid charge ($\geq +6$) [10-14].

184 185 Although the typical V3 loop amino acid sequence is 35 amino acids, V3 amino acid sequences of HIV-1 subtypes and CRFs found in this study showed that all, except three 186 sequences, were 34-amino acids in length. Short length V3 amino acid sequences have 187 188 been reported by other investigators and are frequently associated with CXCR4 usage [5-6]. Sequence conservation in V3 loop was almost entirely consistent when considering the first 189 8 amino acid residues proximal to the terminal Cysteine residue in the base region across 190 191 the subtypes evaluated in this study. It has been reported that sequence conservation in the 192 V3 region across different HIV-1 subtypes is consistent with a V3 interaction with an 193 invariable cellular protein [26]. The sequence variability in the stem and turn regions is as a result of site specific mutations and consequently, most (7/10) of the X4-associated 194 195 mutations detected in this study occur in the stem and turn regions (positions 9-25) of the V3 loop. This observation is consistent with findings by previous researchers and these site-196 197 specific mutations at either side of the V3 loop crown motif are important determinants of 198 CXCR4 coreceptor usage [26, 13].

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200 Some authors have shown that the V3 loop of HIV-1 has a highly conserved crown motif and the GPGQ motif (which was in the majority in this study) in particular, is generally associated 201 202 with African HIV isolates and it occurs irrespective of coreceptor usage [13,16]. However, 203 HIV-1 subtype B has a GPGR crown motif irrespective of coreceptor usage while in HIV-1 204 subtype C, viruses using the CCR5 coreceptor have conserved GPGQ sequence tip and 205 CXCR4 viruses have a substitution which commonly presents as GPGR [20]. In addition, the 206 substitution of GPGQ to GPGL/R/K as observed in 18.8% of the V3 loop sequences of HIV-1 207 subtypes and CRFs in this study, predict CXCR4 tropism, which is in agreement with some 208 studies [27, 28].

209

210 Another determinant of CXCR4 coreceptor usage of HIV-1 isolates which had been reported 211 from previous studies is the presence of basic amino acids at either positions 11 and/or 25 of 212 the V3 loop. Sixteen (94.1%) of the 17 V3 loop sequences with basic amino acids at 213 positions 11 had Arginine residues, while one sequence had Lysine and Arginine residues Formatted: Space Before: 12 pt

214 at positions 11 and 25, respectively. Fouchier and colleagues [25] reported a strong 215 association between the syncytium-inducing (SI) capacity of HIV-1 and the presence of 216 positively charged amino acid residues at positions 11 and 25 of HIV-1 V3 loop, indicating 217 that the V3 genotype may be useful for prediction of the viral phenotype . Also, studies of 3D 218 molecular modeling of the V3 loops from primary isolates whose coreceptor usage was 219 experimentally defined have revealed a charged "patch" on the surface of V3 that correlates with coreceptor usage. This V3 surface patch is positively charged in X4-tropic viruses and 220 221 negatively charged or neutral in R5-tropic viruses, and is formed by two amino acids, at 222 position 11 and at position 24 or 25; amino acids 11 and 24 or 11 and 25 contact each other in 3D space [29]. However, a study showed that sequence variability in V3 loop among 223 subtype C viruses was not associated with the presence of basic amino acid substitutions 224 225 which is in contrast to that observed in subtype B where sequence variability is associated 226 with basic substitutions and is a determinant of altered coreceptor usage[30]. 227

The V3 loop sequence of one sample had a net charge of +6 and multiple amino acid deletions, including the deletion of the N-linked glycosylation site. Some studies have shown that a high net charge of +5 and above in V3 loop with a loss of one or more N-linked glycosylation sites in the C2-V3 region of HIV-1 are characteristics of X-tropic viruses [28, 31-33].

234 Majority (68.8%) of the V3 loop sequences of the HIV-1 strains from this study had one or 235 more X4-associated mutations including the 16 sequences that had only basic amino acid at V3 loop position 11 and/or 25. These X-4 associated mutations are consistent with report 236 237 from previous studies. A review that compared different genotypic coreceptor usage 238 prediction methods and analyses showed that the presence of mutations in the V3 loop is 239 predictive and indicative of CXCR4 coreceptor usage [34]. Sing et al. [14] using univariate 240 analyses for the prediction of HIV coreceptor use from clonal HIV V3 loop sequences 241 identified 41 V3 mutations significantly associated with coreceptor usage. Marcelin and 242 colleagues [35] reported that one reason for the failure of CCR5 inhibitors was the selection 243 of resistance to CCR5 antagonists through amino acid changes in V3 loop.

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Furthermore, other studies have shown that these X4-associated mutations in the V3 loop of 245 246 env and the presence of basic amino acids at V3 positions 11 and 25 are strongly 247 associated with syncytium induction and CXCR4 usage. Chesebro and colleagues in their 248 studies on HIV infectivity and replication in different permissive cells, observed that infectivity and replication in macrophages was affected by the presence of basic amino acids in 249 250 positions 11 and 25 of V3 with viruses harboring such mutations being T-cell tropic and syncytium inducing [10-12]. These observations were also reported by Fouchier and 251 252 colleagues in their studies [25, 36, 37]. Hoffman et al. [38] showed that these basic amino 253 acids in V3 is phenotype associated and results in the use of the CXCR4 coreceptor. Studies 254 on HIV-1 V3 motif analysis by Jensen et al. [5] also observed the association between basic 255 amino acids in V3 and CXCR4 coreceptor usage. Raymond et al. [24] Soulie et al. [39] and 256 Ajoge et al [9] in their various studies also reported that the presence of basic amino acids at 257 V3 positions 11 and 25 was associated with CXCR4 coreceptor usage. 258

259 The 4 N-linked glycosylation sites (NGS) within the C2V3 region are present in different 260 proportions in all, except one, of the sequences. Only 37.5% of the C2V3 sequences had the 261 4 NGS. The loss of one or more N-linked glycosylation sites has been shown to be associated with the development of X4 viruses [23], assist in more efficient use of CXCR4 262 263 and might be an important factor in the switch of CCR5 to CXCR4 viruses, and therefore, virus tropism [40, 41]. Pollaskis et al. [31] also reported that the loss of an N-linked 264 glycosylation site within the V3 region had a major influence on virus switching from R5 to 265 X4 phenotype. 266

267 268 Overall, the predicted coreceptor usage using 4 combined criteria [14, 29] for the V3 loop 269 amino acid sequences of the HIV-1 subtypes and CRFs in this study was 31.2% and 68.8% 270 for CCR5 and CXCR4, respectively, independent of CD4 and viral load status of the individuals, and, irrespective of subtype. The use of the combined criteria has been shown to 271 272 increase both the sensitivity and the specificity of the prediction as reported by Raymond et al. [19] and Ajoge et al. [35]. The high proportion of CXCR4 in this study might be an 273 274 indication that the HIV-1 infected individuals are in the advanced stage of infection 275 associated with accelerated disease progression, or they may be exposed to anti-retroviral 276 therapy, or both [32, 42-44].

The X4 phenotype predominates in both subtype G and CRF02_AG viruses but is not the 278 279 predominant predicted phenotype in CRF06_cpx. The higher usage of CXCR4 by HIV-1G 280 from this study is in agreement with the results of a previous study and further corroborates 281 the evidence that HIV-1G may have a higher proportion of X4 variants [35], although more phenotypic assays for coreceptor usage for subtype G with corresponding genotypic testing 282 283 needs to be done. However, the high usage of CXCR4 by HIV-1 CRF02_AG is contrary to reports from studies by Raymond et al. [17] and Ajoge et al. [35] that reported CRF02_AG 284 HIV-1 strains to be predominantly CCR5-tropic. The prediction on CRF06_cpx shows that 285 individuals infected with this subtype of HIV-1 may benefit from coreceptor antagonists like 286 287 Maraviroc, although there is need for better characterization of HIV-1 CRF06_cpx tropism, 288 and HIV-1G, in Nigeria.

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In addition, 2 (10%) of the predicted CCR5-using HIV-1 from this study had the A22T 290 291 mutation that confers resistance to CCR5 inhibitors like Maraviroc. The high prevalence of 292 X4 tropic viruses and the presence of Maraviroc resistant mutations in some R5 viruses 293 indicate that coreceptor usage ability of patients' viral population will clearly be required 294 before clinical administration of Maraviroc. Moreover, if future phenotypic and genotypic 295 studies confirm the predominance of Maraviroc-resistant CCR5, and CXCR4 phenotypes in 296 Nigeria, a wide scale introduction and clinical application of Maraviroc in HIV-1 treatment 297 and control will be limited. 298

299 4. CONCLUSION

300

This data show a high prevalence of HIV-1 subtype with the GPGQ crown motif in the V3 region suggesting a functional use of this motif. Moreover, X4 viruses predominate in the study population and it is associated with multiple amino acid deletions and mutations in V3 and the loss of one or more N-linked glycosylation sites with some CCR5-tropic viruses showing Maraviroc resistant mutations.

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It will be important to carry out genotypic prediction tests for HIV-1 coreceptor usage in HIV
 patients' population encompassing the various regions of the country and in the predominant
 circulating HIV-1 subtypes before the introduction of CCR5 antagonists for clinical
 management of infected persons in Nigeria.

311

The sequences have been submitted in GenBank with Accession numbers **KF437580**-**KF437619**.

314 CONSENT

315

316 Written informed consent was obtained from every individual whose blood sample was used 317 for the study.

318

319 ETHICAL APPROVAL

320 The University of Ibadan/UCH ethical review board approved the study protocol and written

- 321 informed consent was obtained from every individual whose blood sample was used for the
- 322 study.
- 323

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