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Sepha Nyatichi Mabeya Dip, HND, BSc, MSc, Jomo Kenyatta University of Agriculture & Technology, Dept of Medical Microbiology P.O Box 6200 00200, Nairobi, Kenya. Anthony Kebira Nyamache, Dip, BSc, MSc, PhD, Dept of Microbiology, Kenyatta University P.O Box 43844 00100 Nairobi, Kenya. Caroline Ngugi BSc, MSc, PhD. Andrew Nyerere BEd, MSc, PhD, PDip. Raphael Lihana Wekesa BSc, MSc, PhD, Centre for Virus Research, Kenya Medical Research Institute, P.O Box 54840-00200, Nairobi, Kenya.

Corresponding Author: Sepha Nyatichi Mabeya, Jomo Kenyatta University of Agriculture & Technology, Dept of Medical Microbiology P.O Box 6200 00200, Nairobi, Kenya. Email address: sephakebira@gmail.com or snyatichi@jkuat.ac.ke

PREDOMINANCE OF CCR5 TROPISM IN NON-B HIV-1 SUBTYPES CIRCULATING IN KISII COUNTY, KENYA

S. N. Mabeya, A. K. Nyamache, C. Ngugi, A. Nyerere and R. L. Wekesa

ABSTRACT

Introduction: **The chemokine receptors CCR5 and CXCR4 are considered as the main receptors during HIV infection, replication, transmission and subsequent AIDS progression. CCR5 antagonists are drugs designed to inhibit viral entry by binding to these chemokine receptors. However, characterisation of HIV-1 co-receptor usage before rolling out of CCR5/CXCR4 antagonists has not yet been done in the country.**

Objective: **To determine the HIV-1 co receptor usage among HIV-1 infected individuals and predict possible use CCR5 antagonistic drugs**

Design: **A cross sectional study**

Setting: **Comprehensive HIV care clinics of Kisii Teaching & Referral Hospital, Kenya**

Methods: **A total of seventy-two (72) blood samples were obtained from both drug naïve (32) and experienced (40) study participants. Viral DNA was extracted using QIAamp MinElute Virus kit and partial HIV-1 V3 region was amplified and directly sequenced. Coreceptor usage predicted using *insilico* Geno2pheno (coreceptor) with a false positive rate of 15%**

Results: **Sixty-one individuals (77.8%) were infected with HIV-1 subtype A1, twelve (18.1%) HIV-1 subtype D and four (4.1%) were HIV-1 subtype C. CCR5-using variants were found in 52 (72.2%) while 20(27.8%) participants were infected with CXCR4–using variants. There was no significant difference in co-receptor usage a cross gender, HIV subtypes, disease staging or impact of treatment or CD 4 counts that was observed.**

Conclusions and recommendation: **The detected high level of circulating R5 strains suggests the likelihood of a successful implementation and use of CCR5 antagonists in Kenya where HIV-1 A1 is the most predominant.**

INTRODUCTION

HIV-1 entry into host cells requires coordinated interactions of the envelope glycoprotein gp120 with the CD4 receptor and with one of the chemokine receptors, CCR5 or CXCR4 (1). During early stage of HIV infection, more often the co-receptor CCR5 facilitates primary infection into the host irrespective route of entry and it predominates at early stages of HIV infection (2). However, as the disease progress, the virus may switch to CXCR4 at the later stage of infection. Nevertheless, previous studies have shown that HIV-1 genetic diversity and expose to HAART could influence a viral tropism. It has been should that there is higher prevalence of X4 variants among individuals exposed to HAART than in drug-naive and those infected with HIV-1 subtype D (3-4) This could be facilitated by HAART that has been shown to enhance CCR5 to CXCR4 coreceptor switch (4). However, the dynamics of viral tropism while on drug pressure remains unclear (5). Based on the coreceptor usage, HIV-1 variants thus are classified as CCR5-tropic (R5 variants), CXCR4-tropic (X4 variants), and dual tropic (R5=X4 variants) (3).

With the development of HIV drug resistance, development of new class of drugs is therefore invertible (6). Kenyan government has recently revised its treatment guidelines and introduced the latest ART, INSTIs dolutegravir as one in combination in the first line drugs (7). Following this, there is a need to evaluate the feasibility use of CCR5 antagonists as another new class of entry inhibitors in patient management. The mode of action of these drugs is based on blocking the *env*: CCR5 interaction hence preventing viral entry as its antiviral activity (8). CCR5 antagonists like maraviroc are current being used (8-9) in most developing countries following their success yet, there have not been adopted into the current treatment guidelines in most of the poor resource countries (9). With the feasibility of introduction of CCR5 antagonist in the currently treatment guidelines in Kenyan settings, this study was therefore conducted with the view to map out the cellular tropism of the circulating HIV-1 strains in Kenya. Studies have confirmed that HIV-1 subtype diversity may influence the use of CCR5 antagonists. We therefore also determined the HIV genetic diversity and correlated viral tropisms, currently used antiretroviral drugs and HIV subtypes.

METHODS

This was a cross-sectional study design that consecutively recruited seventy-two (72) individuals from HIV-1 comprehensive clinic at Kisii level five teaching and referral hospital during the period between January and July 2018. The study participants were those found to be HIV-1 infected and attending comprehensive care clinics either on treatment (32) or drug naïve (40) (Table 1). Those who consented, their basic demographic data like age, gender and residence was obtained through a self-structured questionnaire. About 5 ml venous blood was also obtained for CD4 counts, viral tropism and HIV subtyping analysis. Ethically approval was obtained from Kenyatta University scientific ethical committee and permission got from hospital administration before execution of this study.

Viral DNA extraction and HIV-1 C2V3 gene amplification: Approximately, five-millilitre blood samples and viral DNA extracted from blood using QIAamp MinElute Virus kit (Qiagen Inc., Valencia, CA) according to the manufacturers' instructions. The HIV-1 group M *env* gene encoding C2-V3 region (corresponding to 6975–7520 nt in HIV-1 HXB2) was amplified from cDNA based on nested polymerase chain reaction (PCR)

with primers M5 (5¹-CCAATTCCCATACATTATTGTGCCCCAGCTGG-3¹ and M10 (5¹CCAATTGTCCCTCATATCTCCTCCTCCAGG-30) in the first round and M3 (5¹GTCAGCACAGTACAATGCACACATGG-3¹) and M8 (5¹TCCTTGGATGGGAGGGGCATACATTGC-3¹) in the second round (8) according to the manufacturer's instructions. Amplification was conducted under the following conditions, one cycle of 95°C for 10 min and 35 cycles of 95°C for 30s, 55°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min. The PCR amplification was confirmed by visualization with ethidium bromide staining of the gel. The confirmed products from second round PCR were then purified using QIAquick kit (Qiagen) followed by bidirectional population sequencing in automated sequencer ABI 310 (Applied Bio systems, Foster City, CA (6).

Prediction of HIV-1 co-receptor usage: The generated sequences were aligned and edited, using BioEdit software v.7.0.9 (12). Viral tropism was predicted using Geno2pheno (coreceptor) with a false positive rate of 15% (co-receptor) based on 35 amino acids of the V3 region. The 15% false positive rate (FPR) was chosen as cut-off values according to the clinical interpretation scheme based on the European consensus group on clinical management of HIV-1 tropism testing guidelines. The 15% FPR was used as a threshold for X4 predictions (9). The X4 virus was identified when the calculated score of sequences was lower than FPR, while the R5 virus was defined in vice versa. http://coreceptor bioinf.mpi-inf.mpg.de/ (9-10).

CD4⁺ T cell counts: Baseline CD4⁺ T cell counts was performed using a FACS Calibur flow cytometer (Becton-Dickinson, NJ) equipped with automated acquisition and analysis software according to the manufacturer's instructions (11)

Phylogenetic analysis: The obtained sequences from this study were phylogenetically analysed using *insilico* tools. These sequences and their respective reference sequences were aligned using MUSCLE software and later joined using Neighbour-joining method. The phylogenetic tree was then inferred using Tree View (version 1.6.6); Institute of Biochemical and Life Sciences, Scotland, United Kingdom at 1000 data sets bootstrap resampling of multiple alignments. This was performed with aim of testing and confirming the statistical robustness of these trees. Those sequence that were highly hypermutated or divergent were excluded from the analysis (11)

RESULTS

HIV-1 co-receptor usage: HIV-1 co-receptor usage was determined using Geno2pheno (coreceptor) predictor based on V3 amino acid sequences. According to FPR at 15%, the coreceptor usage revealed the existence of CCR5-using viruses in 52 (72.2%) individuals whereas CXCR4-using viruses were observed in 20 (27.8%) individuals. From this analysis, R5-tropisms were therefore confirmed with high predominance in circulating viral strains (Figure 1).

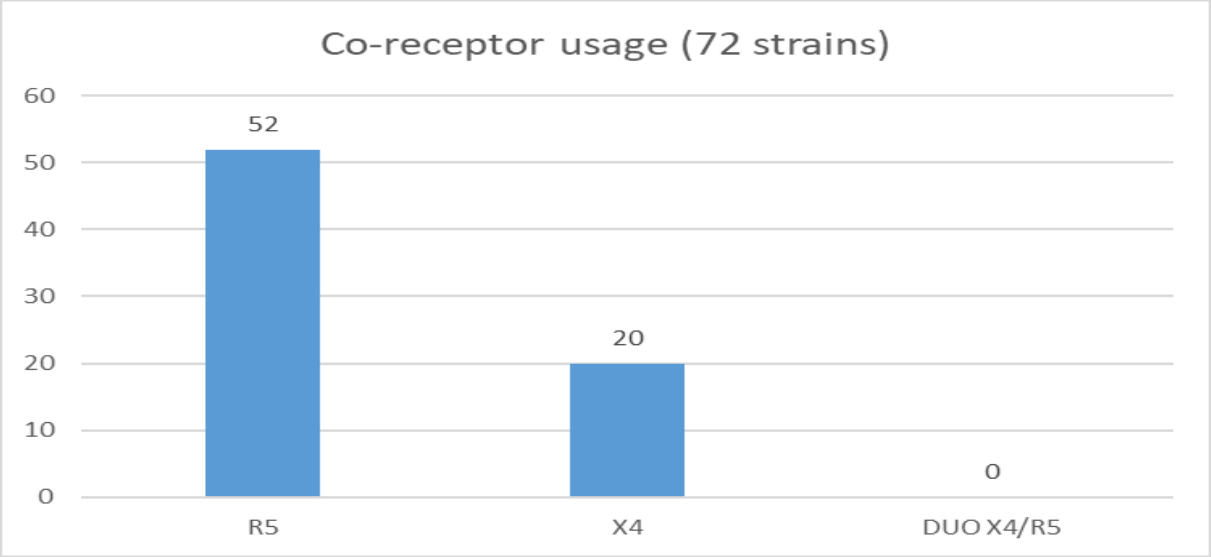

Figure (1) ***In silico*** **viral tropism prediction of the viral co-receptor tropism using Geno2pheno (coreceptor) with a false positive rate of 15% tool. The analysis is applied to 72 primary viral isolates**

HIV-1 subtypes: Analysis of the sequences (72) showed that majority of these sequences belonged to A1 77.8 % (61/72), HIV-1 subtype D 18.1% (12/72) and subtype C 4.1% (4/72) subtypes (Figure 2). Figure 2 shows the phylogenetic tree representing the analysed sequences in conformation of HIV-1 subtypes together with reference sequences from the Los Alamos HIV database. HIV subtyping tools, genotype (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpagex.cgi), Jumping HMMER (http://jphmm.gobics.de/submission_hiv.html) and REGA HIV subtying tool (http://dbpartners.stanford.edu:8080/RegaSubtyping/stanford-hiv/typingtool/) were used in confirming subtyping especially for sequences that had long branches when aligned with a multiple sequencing alignment tree.

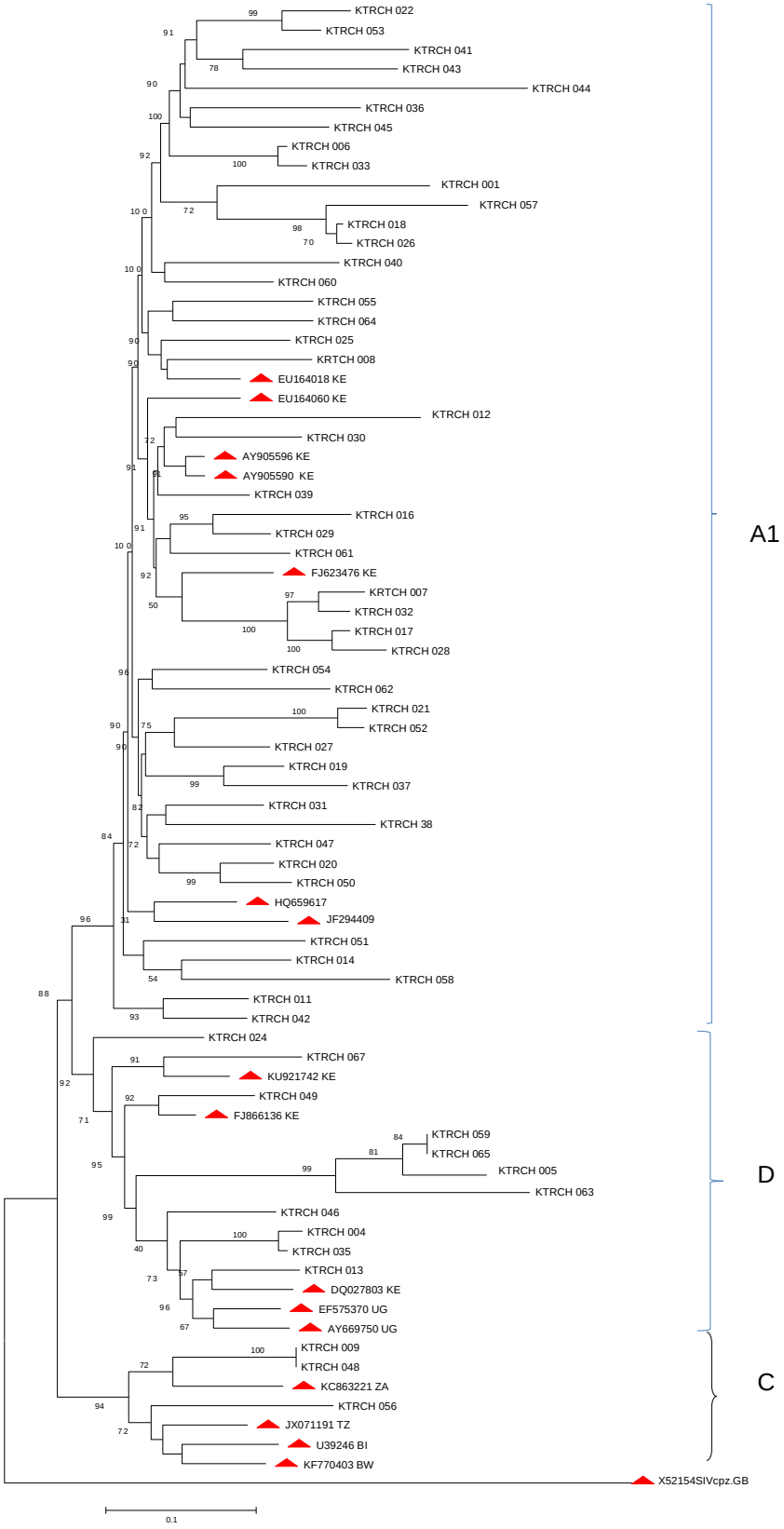

Figure 2: Phylogenetic analysis of *env* sequences generated from sampled drug naive Kisii residents. KEY: The reference sequences indicated by symbol

Table 1
Characteristics of study participants

T cell count	R5 co-receptor usage	X4 co-receptor usage	Female	Male
>400	41	14	30	25
300-400	5	7	8	4
201-300	3	1	1	3
<200	1	0	0	1
CD4 counts	665.9	631.1	652.7	656.7
Gender				
Age (mean Range)			30.9 (5-69)	38.8 (3.5-73)
HIV-1 subtypes				
A1	39	17		
C	2	2		
D	8	4		
Therapeutic regimen				
TDF/3TC/EFV	7	3		
AZT/3TC/EFV	4	4		
DDI/3TC/EFV	4	2		
TDF/3TC/NVP	2	3		
AZT/3TC/NVP	2	0		
DDI/ABC/NVP	1	0		
Treatment naïve	35	5		

Statistical analysis: Correlations of HIV-1 subtype, CD4 count, HIV disease staging and viral tropism were performed and the association between HIV subtypes, CD4 count, gender, disease stage, and viral tropism determined by use of the Chi square test using SPSS v.17software (IBM Company, New York). *p* values less than 0.05 were considered statistically significant. In addition, T- test was used to compare the differences on CD4 counts on the outcome of viral tropisms. Analysis was performed to determine whether there was any significant difference on viral tropism across gender. However, there was no significant difference across gender p=0.945. The CD4 counts and treatment were compared from the studied population in relation to viral tropism. From these findings, there was no difference in CD4 counts in either CCR5 or CXCR4 variants p=0.568 or influence of treatment on viral tropism p=0.31. In addition, disease progression was determined if it could influence the co-receptor usage. However, from the analysis, there was relationship between disease staging and co-receptor usage p=0.175 (Table 3).

Table 3

HIV subtypes and clinical characteristic among patients seeking treatment in Kisii Teaching and Referral Hospital

T cell count	R5 co-receptor usage	X4 co-receptor usage	*p*= value
HIV-1 subtypes			
A1	39	17	
Non-A1	10	6	
CD4 count (mean)	665.9	631.1	0.568
CD4 count (median)	688.0	676.0	
CD4 < 350	44	16	0.346
CD4 ≥ 350	7	6	
Therapeutic regimen			
EFV based ART protocol	15	9	
NVP based ART protocol	5	3	
Treatment naïve	35	5	0.37

DISCUSSION

Previous studies have shown that HIV-1 subtyping A1 is the most dominant virus circulating in Kenya (12-13). This study showed that HIV-1 subtype A1 was the dominant subtype in this part of Kenya with a frequency of 77.8% of the analysed sequences (Figure 2). These findings were consistent with reports from other parts of the Kenya that have continued to confirm the predominance of HIV-1 subtype A1 subtype circulation in the country. From this study, we confirm that Kisii County like rest of the parts of Kenya, AIDS epidemics is mainly driven by HIV subtype A1. The obtained sequences from this study; HIV subtype A1, C or D were clustered with reference sequences from East African countries, Zambia and Botswana showing that viruses were probably of East Africa origin. Phylogenetic analysis of the obtained sequences showed three clusters that were related with previously described sequences from Kenya, more particular HIV-1 subtype A1. However, from this study, no viral recombinants were detected, despite Kisii County among countries that have shown increasing prevalence of new HIV infections.

HIV-1 subtypes

In 2014, the Joint United Nations Programme on HIV/AIDS (UNAIDS) and partners launched the 90–90–90 targets; the aim was to diagnose 90% of all HIV-positive persons, provide antiretroviral therapy (ART) for 90% of those diagnosed, and achieve viral suppression for 90% of those treated by 2020 (14). A crossectional study was conducted in support of this agenda by evaluating the co receptor usage in relation to introduction of CCR5 antagonists. CCR5 antagonists are among the six different classes of drugs targeting HIV at different stages of HIV replication and infection (15). They inhibit CCR5 antagonists like Maraviroc or Vicriviroc are effective drugs in treatment-experienced patients who have viremia with virus using either CCR5 receptor (R5 virus) or CXCR4 (X 4 viruses).

Among the circulating viral strains detected in this study, majority of them were R5 strains, 72.2%. Our data demonstrates high prevalence of R5 using strains among the studied among patients seeking HIV care in Kisii Level Five teaching and referral hospital. This finding concurs with previous studies that have conducted in Kenya and

elsewhere (4,8,16). The detected high levels of R5 strains confirms that majority of the patients were at their early stage of HIV infection. This is in support based on the high CD4 counts measured in addition that most of the patients were drug naïve and those on treatment, had been on drugs in less than six months. This data hence confirms high numbers of circulating R5-using strains in the country.

Previous studies have suggested that R5-using variants found mostly to be the most predominant at early stages of HIV infection (8) HIV-1 usually uses these chemokines and it may switch to X4 –using variants with the disease progression of the disease or treatment (17). This study analysed whether CD4 counts could predict or play a role co-receptor use. The study found out that was no significant relationship between CD4 counts and tropism *p* 0.175. The study also analysed if there was any significant difference in CD4 counts between R5 and X4 variants. There was no significant difference on the levels of CD4 counts *p*- 0.568. The R5 infected individuals had average CD4 count of 656 cells/ml against 631 cells/ml. for X4-using variants (Table 2). Majority of the studies subjects were at early stages of infection. These finding concurs with previous studies that have shown no impact on CD4 count on viral tropism and detected high predominance of R5 using variants (8). However, even in patients with a dominant non-X4 virus, minorities of X4 variants exist (17). In this study, 27.8% were X4-using variants with an average 63,1631cells/ml CD4 count. This suggests that, the X4-infected patients harbours predicted CCR5 inhibitors resistant strains to maraviroc or vicriviroc even though most of the infected individuals with these strains could be at stage 1 of AIDS (4,8).

Analysis was done to compare if treatment had any influence on viral tropism. From the current study, treatment had no influence on viral tropism. Even though this was a crossectional study, these findings confirm previous studies that have indicated that treatment may not influence co-receptor usage. (18) Previous studies have confirmed that different HIV-1 subtypes may vary in the co-receptor usage (19). This may pose a challenge during initiation of treatment especially with the newly introduced new class of drugs CCR5 antagonists. It will therefore appropriate to screen for the co-receptors usage to guide either use of maraviroc or vicriviroc.

Despite these findings, this study had a number of limitations. First, a phenotypic assay was not conducted in the present study due to restricted availability of biosafety facility. However, we used Geno2pheno (coreceptor) with a false positive rate of 15% (co-receptor) based on 35 amino acids of the V3 region, an *insilico* tool which has been confirmed to provide good accuracy in tropism prediction (8). Secondly, population genotypic prediction system used in the current study may results in a misclassification of R5 using variants as X4-using variants. A more sensitive ultra-deep pyrosequencing could have brought multiple orders of magnitudes and even detect minor CXCR44 using variants.

CONCLUSION

Our findings revealed high frequency of HIV-1 R5 tropic strains confirming its predominance circulating variants in this study population. This suggests that, there is a potential benefit with the use of CCR5 antagonists as a therapeutic option in Kenya. In addition to phylogenetic analysis from the present study confirms that like the rest of the parts of the country, HIV-1 subtype A1 remains the most predominant circulating subtype among HIV infected patients in Kisii.

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