

## Viral Subtypes, Coreceptor Usage and Phylogenetic Relationships of Hiv-1 Isolates in Discordant Positive and Concordant Couples

Yohannis Meseret Hambissa<sup>1\*</sup>, Yohannes Mengistu<sup>2</sup>, Rawleigh C Howe<sup>3</sup>, Tsehaynesh Messele<sup>4</sup> and Dawit Wolday<sup>5</sup>

<sup>1</sup>Kotebe University College, P. O. Box 31248, Addis Ababa, Ethiopia

<sup>2</sup>Mauricio's, Botswana

<sup>3</sup>Armour Hansen Research Institute (AHLI), Addis Ababa, Ethiopia

<sup>4</sup>African Society for laboratory Medicine (ASLM), Bole Road, Behind Friendship Building, Bole sub- city, Kebele 02, Addis Ababa, Ethiopia

<sup>5</sup>Medical Biotech Laboratory, P. O. Box 8297, Addis Ababa, Ethiopia

\*Corresponding author: Yohannis Meseret Hambissa, Kotebe University College, P. O. Box 31248, Addis Ababa, Ethiopia, E-mail: [yohannis\\_meseret@yahoo.com](mailto:yohannis_meseret@yahoo.com)

Received date: March 10 2017; Accepted date: April 19, 2017; Published date: April 24, 2017

Copyright: © 2017 Hambissa YM, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

### Abstract

Ethiopian HIV viruses were mainly HIV type C in all discordant positives and HIV/AIDS subjects. But other subtypes such as subtype A, B and recombinant A/G subtypes were also observed. Co-receptor utilization of discordant positive isolated viruses was both CCR5 and CXCR4 in equal proportion. The majority of HIV/AIDS patients used CXCR4, although about one third used CCR5 and a few also used dual co receptors. Our study showed that the majority of subtype C viruses were CXCR4/SI high/rapid subtype. And about one third was CCR5/NSI subtypes. The phylogenetic or evolutionary relationship showed that the majority of the viruses isolated from discordant positives showed sub clustering in one region and those isolated from concordant couples in another region, showing that discordant positive isolated viruses were evolving independently and were related with each other but this was not seen in viruses of concordant couples and HIV/AIDS subjects.

### Key words:

Gut-kidney axis; Celiac disease; IgA nephropathy; Berger's disease; IgA deposit; tissue transglutaminase; Gluten

### Introduction

Ethiopia, located in Eastern Africa, is one of the Sub-Saharan countries in which HIV infection is mainly due to subtype C, although sub-clustering analysis showed a slightly different form designated by "C" [1]. Although specific subtypes tend to predominate in specific regions, due to the pattern of transmission and population migration, the proportion of subtypes in defined population are not stable. The appearance of recombinant forms has also further complicated the sub-type distribution of HIV specific subtypes [2].

HIV as other retroviruses can exist in two forms, both as RNA within virus particles and host-cell associated DNA. Molecular viral sequencing from both sources can depict so many valuable properties of the virus. For example, chemokine utilization properties can be determined from the viral sequence and this can also help us to determine the nature of the virus. Chemokine receptors are down or up-regulated depending on the activation status of the cell and this may enhance or reduce susceptibility to HIV infection [3,4]. Coreceptor tropism has been established as a determining of disease progression [5]. Individuals with virus using the CCR5 coreceptor generally have a slower rate of progression and lower viral load [5]. However, the presence of mixed/dual-tropic CCR5/CXCR4 population or CXCR4-using virus is more common at lower CD4 cell counts and higher viral loads and aggravated disease condition [6].

No study was carried out on molecular sequencing of co-receptor utilization and the type of virus in Ethiopia. Recent distribution of HIV subtypes are also missing. The viral type and relationships of HIV in discordant and concordant HIV positives are not known. This study addresses these issues.

### Methods

#### Sample collection and peripheral blood mononuclear cell isolation

Blood samples were collected from all over Ethiopia. In short, venous blood was collected from the study subjects in EDTA vacutainer tubes and serum, buffy coat and blood cells were separated by centrifugation. The serum and buffy coat samples were separated and stored at -80°C until further analysis was carried out. The remaining blood cells were diluted with PBS and layered over Ficoll-Hypaque. After density gradient centrifugation on Ficoll-Hypaque, PBMC was collected and viable frozen in liquid nitrogen until further analysis was carried out. Serum, buffy coat sample and PBMC were transported to Australia and analysis was done at Center for Clinical Immunology and Biomedical Statistics laboratory in Perth, Western Australia.

#### Full length sequencing of the HIV genome

Methods for each step of sample preparation, including reverse transcription, PCR reactions, and sequencing were developed on high-throughput automated liquid handling instruments (Biomek FX, Beckman Coulter) with 96/384 well capacity. Briefly, depending on viral load, HIV-1 RNA was isolated from 0.5-1 mL of plasma, RNA was

then reverse transcribed to generate a template for full length amplicons of the HIV genome. Nested PCRs were performed to generate 2-3 amplicons spanning the genome. Both strands were sequenced and sequencing data was collected on an ABI prism 3100XL Genetic Analyser. Sequencing software Analysis Software Assign Viral (Conexio Genomics Pty Ltd) was used for the data analysis. Alternate primers were used where necessary to fill gaps remaining in the sequence. A software program "Primer Sleeper" automated selection of alternative primers was used based on the sequence already generated.

Full and near-full genome sequencing was carried out by comparative analysis by aligning sequences to HIV reference sequence, HXB2 [7]. Where anomalies have been identified in each region they were listed along with the position. All anomalies have been checked against the sequence data and verified and checked as present. All sequence data has been verified and checked against HXB2. All data were generated by using proviral DNA and by sequencing from RNA.

Genomic DNA was obtained directly from the patients peripheral blood mononuclear cells (PBMCs) -buffy coats. Purified HXB2 DNA served as a template for sequencing; taking into consideration that the profiles of viral variations across the HIV-1 genome were similar among subtype B and C. Both strand sequencing was combined with a strategy involving overlapping sequences. Dye terminator sequencing on an automated DNA sequencer (model 373A; Applied Bio systems, Inc., Foster city, Calif) was used. Thus, overlapping polymerase chain reactions (PCRs) were done to obtain the full- length and the near-full-length genome sequence of each strain.

Near full-length proviral genomes were amplified as previously reported by Gao et al. [8] The complete genome sequences were determined by the primer walking method on both strands of DNA and aligned with a set of reference sequences, using the profile alignment option of CLUSTAL W [8].

Each sample was amplified by a two-round PCR amplification reaction and all PCR fragments were detected by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The amplified products were purified using a QiaQuik gel extraction (Qiagen, Qiagen SA, France).

Nucleotide sequences were obtained by direct sequencing of the amplified DNA, using the inner primer of the *nef* gene, the inner primers of the accessory genes, and several primers encompassing the *gag* and the *pol* genes. Cycle sequencing was performed using fluorescent dye-terminator (dye terminator cycle sequencing with Ampli Taq DNA polymerase FS, Perkin Elmer, Roissy; France) according to the instructions of the manufacturer. Electrophoresis and data collection were done on an Applied Biosystems 373A (stretch model) automatic DNA sequences. The sequence fragments were assembled into contiguous sequencer and a consensus of the two strands was formed by using the Seqed program (Applied Biosystems, Branchburg, NJ).

The PCR conditions for each round of amplifications were as follows: a first denaturation step for 5 min at 94°C, 30 sec at 55°C, and 2 min at 72°C, with a final extension for 5 min at 72°C, in a final volume of 50ul; 20Pmol/lit of each primer, 20 mmol/lit of dNTPs, and 2.5 U of Taq polymerase were used. Of this round 5 µl was used for the second round, which used 32 cycles with 50°C as the annealing temperature. This protocol was used for all viruses. Finally, these were separated by electrophoresis in 5% acryl amide gels under non-denaturing conditions at 150V for 4 h, and then visualized by autoradiography.

## Blast subtyping

A web-based HIV-1subtyping system that uses the BLAST algorithm was done on our envelope gene sequence to identify subtypes [9]. The subtyping method employs a BLAST comparison between the HIV-1 sequence to be sub typed, input sequences, and a panel of complete genome references for the subtypes A, B, C, D, E, F, G, and H of group M, as well as for group O and N available in the GenBank. This program detects the best local similarities between a query sequence and a set of HIV-1 subtype reference sequences without performing a global alignment. A sliding window along the query sequence allows the detection of possible intersubtype recombinants with interspersed regions from two or more subtypes.

## Phylogenetic tree analysis

The phylogenetic neighbour-joining tree was generated establishing distances between sequences using the phylogentic computerized program. Nucleotide sequences for each of the genes were aligned using CLUSTAL W [10] with minor manual adjustments, bearing in mind the gene sequence sites where there was a gap in any of the sequences, as well as areas of uncertain alignment, were excluded from all sequence comparison. The phylogenetic tree was constructed from nucleotide sequences, using the neighbour-joining method and the Kimura two-parameter model. That is, genetic distances were calculated with Kimura's two parameter method [11]. The bootstrap values at each node represent the percentage of 1000 bootstrap replication that supports the branching order.

Analyses of envelope C2V3 hypersensitive loop was also carried out to analyze co-receptor usage and percentage of viruses using CCR5 and R4 was determined for HIV positive discordant couples and for HIV positive concordant couples.

## Result

### Viral genotyping

To investigate whether the difference between discordant positives and concordant couples was due to viral genetic factors or not, partial and full genome sequencing was carried out on HIV isolates from discordant positives, concordant couples and HIV/AIDS patients as indicated under material and method. Full or near- full length genome sequencing was carried out from proviral DNA and from viral RNA, by comparative analysis by aligning sequences to HXB2, and by amplifying using PCR techniques, as indicated under material and method.

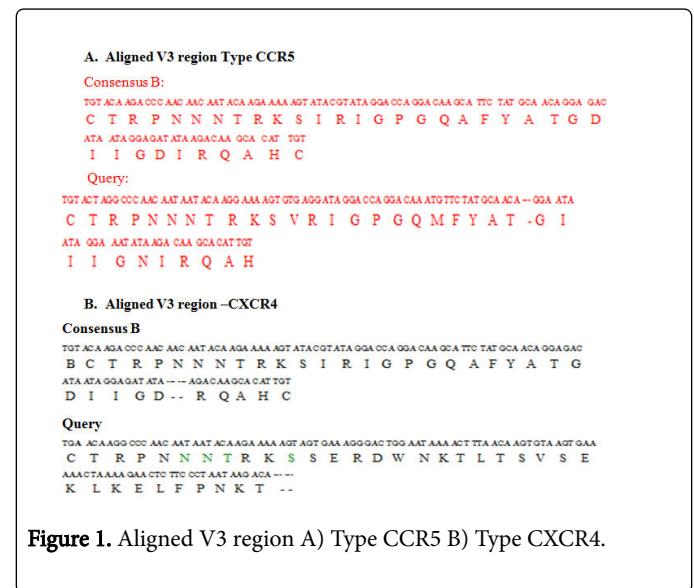
Out of the total full and near-full length viral sequences, 9 full genome sequence of viruses isolated from discordant positive, 2 full genome sequence of viruses isolated from concordant couples, and 24 full genome sequences of viruses isolated from HIV/AIDS patient isolated viruses were analyzed using different methods for their viral subtype, co-receptor usage and geographical similarities with other viruses from different countries. Table 1 summarizes the results obtained from these analyses.

HIV *Env* gene sequence based sub typing was carried out using three web-based: Seq Locator Ianl [12], Raga HIV-1 sub typing Tool-version 2.0 [13] and HIV-1 automated HIV blast Ianl [14]. Results were accepted when there was agreement between the results of the three and rejected or considered as ambiguous when there was disagreement between the three results.

No.	Id	DSCP, CONC and HIV/ AIDS	Subtype	Co- receptor	Lanl Geograp hy	Remark
1	AKC14	CONCP	C	Ambiguo us	DJ, IS	
2	AKC13 9	DSCP	Ambiguo us			
3	ARC15	DSCP	C			
4	AWA3	DSCP	C		BW, MW	
5	AWA7	DSCP	C			
6	AWA9	DSCP	C			
7	AWA10	DSCP	C			
8	AWA18	DSCP	C			
9	AWA25	DSCP	C	CCR5		
10	AWA32	CONC	C			
11	BCT10	DSCP	A	CXCR4	Ug, KE, TA	
12	ET002	HIV/ AIDS	B	CXCR4		
13	ET003	HIV/ AIDS	C	CXCR4		
14	ET004- 1	HIV/ AIDS	C(wt)	R5X4	C, IN	very similar to HXB2
15	ET004- 2	HIV/ AIDS	C	CXCR4	DJ, FL, ZA	
16	ET005	HIV/ AIDS	C	CXCR4	ZM	
17	ET006	HIV/ AIDS	C	CXCR4		
18	ET007	HIV/ AIDS	C		ZM,MW, BW	
19	ET008	HIV/ AIDS	A or A/G	CXCR4		
20	ET009	HIV/ AIDS	C			
21	ET010	HIV/ AIDS	C			
22	ET011	HIV/ AIDS	C			
23	ET012	HIV/ AIDS	C			
24	ET013	HIV/ AIDS	C	CXCR4		
25	ET014	HIV/ AIDS	C	CXCR4		
26	ET016	HIV/ AIDS	A or A/G	CCR5		

27	ET018	HIV/ AIDS	A or A/G	CXCR4		
28	ET022	HIV/ AIDS	C	CXCR4		
29	ET024	HIV/ AIDS	C	R5X4		
30	ET031	HIV/ AIDS	C			
31	ET052	HIV/ AIDS	C	CCR5		
32	ET060	HIV/ AIDS	C	CCR5		
33	ET062	HIV/ AIDS	C	CCR5		
34	ET064	HIV/ AIDS	C	CCR5		
35	ET068	HIV/ AIDS	C	CCR5		

**Table 1.** HIV subtypes and Co-receptor utilization by these viruses. DSCP= discordant positives, CONC=concordant couples, HIV/ AIDS=HIV AIDS patients. All samples were obtained from Ethiopian regions (ET=Ethiopia, AWA=Awassa, BCT=Bahir Dar central clinic, AKC=Akaki Kaliti clinic (Addis Ababa). ARC=Arada clinic (Addis Ababa)



**Figure 1.** Aligned V3 region A) Type CCR5 B) Type CXCR4.

HIV blast Ianal was also used to locate the geographical similarity of the subtypes with other countries' or continent's subtypes. The result was as shown in Table 1 and Table 2. In discordant positives 77% (7/9) were subtype C while 11% (1/9) was subtype A. Recombinant forms were not observed in discordant positive subjects. In concordant couples all, 100% (2/2) were sub-type C, a virus subtype which is a predominant form in Ethiopia. In HIV/AIDS patients the majority 79% (19/24) were subtype C subtypes. But other subtypes such as subtype B (4.1%; 1/24) and recombinant A or A/G forms 12.5% (3/24) were also found (Table 2).

Lanl geographical distributions showed that many of these viruses were very similar to other African country's subtypes (Table 1). Some of these countries were neighboring countries such as Djibouti, Kenya, Uganda, and many East and Central African countries such as Tanzania, Zambia, Zimbabwe; and South African countries such as Malawi and Botswana. Similarity was also observed between Israel and Ethiopian subtype sequence in one patient (Table 1). In one case the wild type viral sequence was also observed.

The observation of other subtypes such as subtype A and B and the recombinant forms than were previously observed subtype C and C' showed that other viruses, other than type C were introduced or being introduced to Ethiopia. The emergence of recombinant forms also showed that people are acquiring multiple viruses and new forms are still appearing.

Viral co-receptor usage was also analysed by using web-based, WebPSSM, gene2pheno (genotype (version.0)), as previously described by Fouchier et al. [15], aimed at revealing the relationship between V3 loop sequence and viral coreceptor usage. Ethiopian HIV viruses used CCR5 (Figure 1A) and CXCR4 (Figure 1B) co-receptors. Others which used R5X4 were also observed. Table 1 and 2 shows the types of viruses using CCR5, CXCR4 and R5X4 co-receptors and their proportions.

HIV discordant positive subjects used CCR5 and CXCR4 co-receptors in equal proportion (Table 2). Their ratio was 1:1. Significant proportion of HIV/AIDS viruses (61% (11/18) used CXCR4 and about a third, 33.3(6/18) used CCR5 co-receptors (Table 2). About 5.7% (2/18) used dual co-receptors (Table 2). The ratio of CXCR4 to CCR5 was almost 2:1.

Parameters	DSCP (%)	CONC (%)	HIV/AIDS (%)	Total Isolates
Subtypes A A orA/ G(recombinants)	11 (1/9)			9
	0		12.5 (3/24)	24
	0		4.1 (1/24)	24
	77 (7/9)	100 (2/2)	79 (19/24)	35
Co-receptors CCR5 CXCR4 R5X4	50 (1/2)		33.3 (6/18)	20
	50 (1/2)		61 (11/18)	20
	0		5.7 (2/18)	18

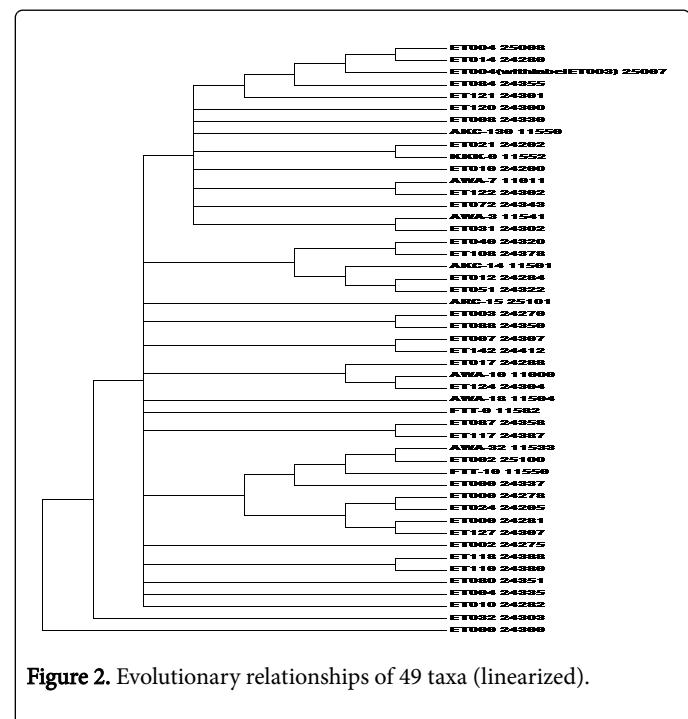
**Table 2.** Percentages of different subtypes and Co-receptor types. DSCP: Discordant Positive; CONC: Concordant Couples; HIV/AIDS: HIV AIDS Patients.

To see the phylogenetic relationship, or their evolutionary relations, of viruses isolated from discordant positives, concordant couples and HIV/AIDS patients, a complete genome nucleotide sequence tree of 49 isolates was constructed, as indicated under material and method. The phylogenetic tree was constructed from the nucleotide sequence of 6 discordant positive, 5 concordant couples and 38 HIV/AIDS subjects isolated HIV viruses (Figure 2).

As indicated in Figure 2, the discordant positive HIV isolates (AKC 139, KKK-6, AWA-7 and AWA-3) subclastered in one region. The other two isolates of discordant positives (ARC-15, AWA-18 and AKC139) diverged early from a common stock and are evolving independently without branching and rebranching from others. Three

isolates (KKK-6, AWA-6, and AWA-7) formed a branch from a common stock and diverged from HIV/AIDS patient's isolated stocks. KKK-6 diverged from a common stock and separated from ET021 at a time. Similarly, AWA-7 diverged from a common stock with ET122 and separated from it at a certain time and is evolving on its own. AWA-3 also diverged from a common stock with ET031 and is evolving on its own.

A similar sub clustering of another DSCP isolates was observed in discordant positives at opposite pole, showing that discordant positive isolates were diverging very far from each other. There was a sub clustering by isolates FTT-19, FTT-6, AWA-32, AWA-16 and AWA-18. AWA-32 and FTT-16 diverged early from a common stock and are evolving independently. AWA-32, FTT-10 and FTT-19 separated or rebranched recently from a common stock and are evolving separately from HIV/AIDS isolates. AWA-18 and FTT-8 evolved independently and are evolving in parallel with each other. In CONC isolates (AKC14 and AWA32) no tendency of subclustering was observed as in HIV/AIDS subjects



**Figure 2.** Evolutionary relationships of 49 taxa (linearized).

HIV/AIDS isolated viruses were distributed all over and did not show sub clustering. Very few (4 out of 38) evolved independently, while 4 out of 9 separated from the main line and were evolving independently in discordant positive isolated HIV. Like HIV/AIDS isolated viruses fewer (2 out of 6) were evolving independently in concordant couples. More recent branching from a common stock and rebranching was observed in HIV/AIDS isolated viral stocks, showing more divergence still in this group.

The evolutionary history was inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method

and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 71 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

## Discussions

HIV is characterized by its enormous plasticity and non-stoppable diversity due to error-prone reverse transcriptase and absence of exonuclease editing the errors occurred. Uniformity in genetic make-up is never the rule in HIV. HIV clades are phylogenetically classified on the basis of the 20-50% differences in envelope (env) nucleotide sequences into three groups; group M being one of them. Within M subgroups, inter-clade env variation differs by 20-30%, whereas intra-clade variation of 10-15% is observed [16].

Geographical distribution of the different subtypes is very heterogeneous and in Africa all subtypes have been extensively characterized by genetic analysis. The various genetic subtypes differ in their geographic spread and so the subtype designations have been powerful molecular epidemiological markers for tracking the course of the global pandemic.

HIV-1 subtype has been estimated to account for 48% of HIV-1 infection worldwide and 51.5% of HIV-1 infection in Africa [17], where the main mode of transmission is heterosexual. The predominant subtype of HIV in Ethiopia is also subtype C since the start of the epidemics over two decades of age. But sequence analysis demonstrated the presence of distinct subcluster (C prime C') within the main subtype group of viruses in Ethiopia. A number of recent findings [18,19] argue that one possible cause of the high viral diversity in Sub Saharan Africa could be higher flexibility of subtype C virus and its altered ability to diversify. The arguments as cited by [19] include: that Subtype C is predominant in most recent HIV-1 epidemic worldwide; the highest prevalence of HIV-1 infection in various epidemics is caused mainly by subtype C; Subtype C virus may have a faster disease progression; patients infected with HIV-1 subtype C develops to AIDS earlier than patients with subtype A virus; and the viral load of subtype C infections may be higher in different compartments that might cause an increased level of viral transmission.

Indeed, clade C has created the recent epicenter of HIV pandemic by its uncontrolled spread throughout Botswana, Zimbabwe, Malawi, Zambia, and Namibia, Lesotho, South Africa, India, Nepal and China. Ethiopian clade C isolates differ (with respect to RT) from clade B by 6.8-10% and intra-clade differences of 3.5-5.8% have been reported for strains from Africa, India and South America [20].

In light of all these features of subtype C, the Ethiopian HIV is characterized by the predominance of this subtype C as observed from our findings. In all, discordant positives, concordant couples and HIV/AIDS patients the majority of the viruses isolated were subtype C, although there was an introduction of subtype A, B and recombinant forms. It was not clear from this study whether the subtype C from discordant positives, concordant couples and HIV/AIDS were the same subtype C. Analysis of the gag and pol genes in previous studies showed that Ethiopian HIV virus can be classified into C and C' (1). It is also not known whether these viruses have different biological properties or not. It is possible that the viruses from discordant positives might have been Subtype C' as our previous results indicated that discordant positive subjects were not the same as HIV/AIDS

patients or concordant couples in many of their profiles including lower CD4 count and higher viral load and others.

The predominance of subtype C in the Ethiopian epidemics was still maintained as indicated by the C subtype observed in this study and other previous reports. Thus, our result agrees with previous findings that Ethiopian HIV is predominantly subtype C. What was different from previous study was that in discordant positives the same virus which caused AIDS epidemic was found in large number. But further characterization of pol and gag and amino acid sequences should be carried out to show whether these viruses were subtype C' and were different in biological properties from HIV/AIDS isolated viruses.

Another difference in our study from previous studies was the observation of subtypes A and B and recombinant forms from Ethiopia. This is possible because as HIV/AIDS pandemic grows, viral strains become more geographically dispersed and simultaneous presence of multiple subtypes all over the world is becoming very common. It is expected that this will increase due to population migration and the expansion of trade and investment. Since subtype A is very common in neighbouring and sub-Saharan countries and subtype B is common in European and American, these might have been introduced to Ethiopia.

The recombinant forms observed were the ones which were very common in Africa, A/G, and the group which also used CCR5 and CXCR4 co-receptors (21/22).

Recombinant events among sequences of different genetic subtypes of HIV group M have been frequently identified and are becoming of epidemiological importance. The observation of recombinant forms in Ethiopia indicated that different subtypes were circulating in the population simultaneously. Thus, the finding of subtype A or A/G indicated that either type C type A and G were also circulating in the population. The number of different subpopulations and recombinant forms circulating in Ethiopia might be greater than that reported and further molecular epidemiological studies are needed to show the real situation. Overall, although subtype C virus is the predominant form, other subtypes are also creeping in to the country and recombinant forms are also being observed.

The V3 loop of HIV-1 is critical for co-receptor binding and is the main determinant of which of the cellular co-receptors, CCR5 or CXCR4, the viruses use for cell entry [21]. Most HIV clades cause disease by assuming the CCR5+/NSI phenotype during early disease and the CXCR4/SI phenotype during the end stage of the disease [22]. Thus, it has been shown that viruses binding to CCR5 or CXCR4 are almost exclusively present during the early asymptomatic stage of infection, whereas CXCR4-binding viruses may emerge in later phases of the infection and are associated with a CD4+ T cell decline and progressing towards AIDS. Relatively CCR5/NSI strains are more conserved when compared to more diverse CXCR4-trophic and SI strains evolving in an apparently unconstrained manner.

The view that early viruses use CCR5 and late viruses use CXCR4 depends up on the type of the clade [23] and may not hold true for clade A, C or D. Clade A viruses tend to favor CCR5 even at later stages, while clade C strains rarely become CXCR4/SI even in the stage of the disease [24]. The reason for this was attributed to persistent immune activation experienced by many Africans [18], as persistent immune activation constantly trigger CCR5 over-expression. Thus, the CXCR4-positive rapid/high phenotype is underrepresented among subtype C isolates and syncytium-inducing phenotype is rare among subtype C-infected patients [19].

HIV subtype C was the predominant subtype and accordingly CCR5 would have been used as a coreceptor for viral entry into the cell. However our result showed even in discordant positives CCR5 and CXCR4 were used equally (50% each). In HIV/AIDS patients about 61% (22/18) used CXCR4 as a coreceptor and only 33.3 % (6/18) used CCR5 coreceptor, the remaining 5.7% (2/18) used dual coreceptors. Our result does not agree with the previous work that subtype C viruses never use CXCR4 even in late stage of the disease [25]. Our concordant couples and HIV/AIDS patients (subjects which were not couples but AIDS patients for which we did HIV sequencing) showed the characteristic AIDS feature with devastating diseases and hence were CXCR4/SI rapid/high type. But discordant positive subjects showed normal CD4 count and lower viral load and used both CCR5 and CXCR4 equally. The coreceptor usage in HIV/AIDS patients was not homogeneous but both coreceptors were used in a ratio of 2:1 of CXCR4 to CCR5. The reason for this was not clear from this study but it was obvious that the Ethiopian subtype C isolates used (probably converted from CCR5 to CXCR4) CXCR4 during AIDS stage. The molecular biological identification of these receptors was also different from our immunological staining, which showed about 8-10% CCR5 coreceptor only, probably indicating the staining method might have not been a method of choice when compared to this novel molecular method. Ethiopian HIV is the worst HIV and its progress to AIDS is rapid as was shown by previous workers. This is the characteristics of CXCR4/SI using rapid/high strains and our result reflected this feature of HIV virus. But the fact that about a third of these viruses used CCR5 coreceptors indicated that Ethiopian HIV viruses were/are not homogeneous in their coreceptor utilization. Thus, it is tempting to stress that further molecular epidemiological studies involving larger sample size should be conducted.

To understand more fully if there was genetic variation among isolates obtained from discordant positives, concordant couples and HIV/AIDS subjects, phylogenetic trees were constructed using neighbour-joining algorithm and the Kimura two-parameter model. A complete genome nucleotide sequence tree depicting the phylogenetic positions of the 49 characterized HIV-1 strains were used (Figure 2).

Phylogenetic analysis showed that most viruses identified from discordant couples formed sub cluster around the same region, showing that these viruses were more closely related to each other and were of recent origin or emerged together. Two isolates from discordant group (AWA-18 and ARC-15) diverged early and were evolving independently without diverging to any kind, showing independent evolution. But AWA-18 was far from AR-15 on the evolutionary tree. AWA-18 evolved in parallel with FTT-8 which was a concordant subject. Some of the viruses of discordant positives diverged from a common stock, which gave rise to both discordant positives and HIV/AIDS derived viruses, showing that different lines of evolution being pursued probably one to a mild form and the other to an aggressive one.

Similar sub clustering was also observed in viruses isolated from concordant couples, although at a distant site on the evolutionary tree, showing viruses isolated from discordant couples were different from concordant couples on the evolutionary tree. HIV/AIDS isolated viruses did not form sub clustering and highly divergent groups, suggesting a more long-standing evolution of these groups sometimes giving rise to discordant positive viruses. Many of them started evolve independently early and were evolving in to a separate line.

## Conclusions

Ethiopian HIV viruses were mainly HIV type C in all discordant positives and HIV/AIDS subjects. But other subtypes such as subtype A, B and recombinant A/G subtypes were also observed. The observation of other subtypes and recombinant forms indicated that other subtypes are circulating in the population and the possibility of co-infection by different subtypes. Co-receptor utilization by Ethiopian HIV subtype C was different from the previous co-receptor utilization by subtype C viruses. Co-receptor utilization of discordant positive isolated viruses was both CCR5 and CXCR4 in equal proportion. The majority of HIV/AIDS patients used CXCR4, although about one third used CCR5 and a few also used dual co receptors. Our study showed that the majority of subtype C viruses were CXCR4/SI high/rapid subtype. And about one third was CCR5/NSI subtypes. The reason for this mosaic utilization of co-receptors was unknown from this study although the majority of our subjects showed a typical AIDS profile. The phylogenetic or evolutionary relationship showed that the majority of the viruses isolated from discordant positives showed sub clustering in one region and those isolated from concordant couples in another region, showing that discordant positive isolated viruses were evolving independently and were related with each other. HIV/AIDS isolated viruses did not show subclustering and were highly divergent. Some also segregated early and were evolving independently.

## References

1. Abebe A, Pollakis G, Fontanet AL, Fisseha B, Tegbaru B, et al. (2000) Identification of a genetic subcluster of HIV type 1 subtype C (C') widespread in Ethiopia. *AIDS Res Hum Retroviruses* 16: 1909-1914.
2. Tripathi P, Agrawal S (2007) The role of human leukocyte antigen E and G in HIV infection. *AIDS* 21: 1395-1404.
3. Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, et al. (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382: 829-833.
4. Furrer H, Wendland T, Minder C (1998) Association of Syncytium inducing Phenotype of HIV-1 with CD4 cell count, viral load and sociodemographic characteristic. *AIDS* 12: 1341-1346.
5. Bozzette SA, McCutchan JA, Spector SA (1993) A cross- sectional comparison of Persons with syncytium and non-syncytium- inducing human immunodeficiency virus. *J Infect Dis* 168: 1374-1379.
6. Ratner L, Haseltine W, Patarca R, Livak KJ, Starcich B, et al. (1985) Complete nucleotide sequence of the AIDS virus HTLV-III. *Nature* 313: 277-284.
7. Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, et al. (1999) Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* 397: 436-441.
8. <http://www.ncbi.nlm.nih.gov/retroviruses/HIV-1>
9. Thompson JD, Higgins DG, Gibson TJ (1994) CLUTAL W -improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap Penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680.
10. Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16: 111-120.
11. [www.hiv.lanl.gov/contents/sequence/BAS](http://www.hiv.lanl.gov/contents/sequence/BAS)
12. Fouchier RA, Groenik M, Tessmette M (1992) Phenotype associated sequence variation in the third variable domain of HIV-1 gp120. *J Virol* 66:3183-3187.
13. Fields BN (1990) Virology, 2nd edition: Raven Press Limited, New York, pages 1529-1570.
14. Essex M (1998) State of the HIV pandemic. *J Hum Virol* 1: 427-429.

15. Fahey JL, Taylor JM, Manna B, Nishanian P, Aziz N, et al. (1998) Prognostic significance of plasma markers of immune activation, HIV viral load and CD4 T-cell measurements. *AIDS* 12: 1581-1590.
16. Novitsku VA, Montano MA, McLane MF (1999) Molecular Cloning and Phylogenetic analysis of Human Immunodeficiency Virus Type 1 Subtype C: a set of 23 Full-length Clones from Botswana. *J Virol* 73: 4427-32.
17. Redenbarg CM, Li Y, Trask SA (2001) Near Full Length Clones and Reference sequences for subtype C Isolates of HIV Type 1 from Three Different Continents. *AIDS Res Hum Retro* 17: 161-168.
18. Sierra S, Kupfer B, Kaiser R (2005) Basics of the virology of HIV-1 and its replication. *J Clin Virol* 34: 233-244.
19. Worbey M, Bjork A, Wertheim JO (2007) Point, Counterpoint: The evolution of pathogenic viruses and their human hosts. *Annu Rev Ecol Evol Syst* 38: 515-540.
20. Peeters MF, Liegeois N, Torimiro N (2000) Characterization of a highly replicative intergroup M/O human immunodeficiency virus type1 recombinant isolated from a Cameroonian patient. *J Virol* 73:7368-7375.
21. Tscherning C, Alaeus A, Fredriksson R, Björndal A, Deng H, et al. (1998) Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. *Virology* 241: 181-188.
22. Pao D, Fisher MS (2005) Transmission of HIV-1 during Primary infection: relationship to sexual risk and sexually transmitted infections. *AIDS* 19: 85-90.
23. Lehman DA, Farquhar C (2007) Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. *Rev Med Virol* 17: 381-403.
24. Johnston E, Jimenez J, Munteha J (2003) High frequency of Syncytium inducing and CXCR4 tropic viruses among HIV-1 subtype C infected patients receiving Antiviral treatment. *J Virol* 770: 7682-7688.
25. Sallusto F, Lenig A, Förster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

This article was originally published in a special issue, entitled: "**HIV-1 Vaccine Immunology**", Edited by Dr. Hung V. Trinh, Walter Reed Army Institute of Research, USA