

**HIV-1 SUBTYPE EPIDEMICS IN ETHIOPIA:  
AN ASSESSMENT OF THE PREVALENCE OF NON C-SUBTYPES  
IN ADDIS ABABA.**

**A THESIS SUBMITTED  
TO  
THE SCHOOL OF GRADUATE STUDIES  
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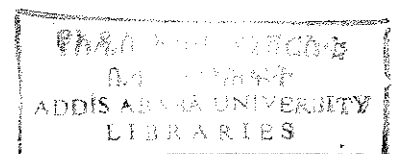
**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF  
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**BY**

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## ABSTRACT

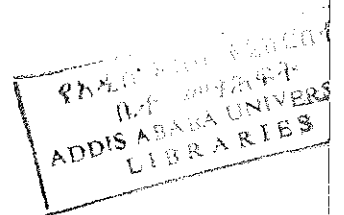
The present study was conducted to assess the possible influx of new HIV-1 subtypes into the population of Addis Ababa, Ethiopia. In an unlinked anonymous cross sectional study, 150 commercial sex workers (CSW) reporting in 1997 to two STD clinics in Addis Ababa- Kazanchis and Tekle-Haimanot were enrolled. For HIV-1 subtyping, the WHO algorithm of peptide ELISA followed by heteroduplex mobility assay (HMA) and DNA sequencing was carried out. The HIV-1 prevalence among the CSW was 44.7% (67/150). Peptide ELISA identified 46.3% (31/67) plasma samples to be reactive with subtype C peptides; 30% (20/67) to be reactive with both subtype C and A peptides; 3% (2/67) to be reactive with peptides A, C and D; 1.5% (1/67) with peptides B and D and 1.5% (1/67) with peptides C and D. Six (9%) samples were indeterminate (OD just below cut-off) and another 6 (9%) were non-reactive. Subtyping performed with HMA identified 86.5% (58/67) as subtype C, 6% (4/67) as subtype A and 1.5% (1/67) as subtype D. Four plasma samples remained untypable using this technique. DNA sequencing of the gp120 V3 regions of 8 samples (2 subtype C, 3 subtype A, 2 untypable and 1 aberrant sample by HMA) clustered all in subtype C. One subtype D sample by HMA was found to belong to the same subtype by sequencing. Four samples remained untypable by sequencing. In general, 95.5% (64/67) were subtype C, 1.5% (1/67) subtype D and 3% (2/67) were untypable. In conclusion, although this study provides evidence for influx of a non-subtype C HIV-1 strain (D) into Addis Ababa, the far majority of HIV-1 infections in this city remain of subtype C.

# 1. INTRODUCTION

Since the discovery of human immunodeficiency virus type one (HIV-1) and type two (HIV-2) as the causative agents of acquired immunodeficiency syndrome (AIDS) (Gallo et al., 1984 and Levy et al., 1984), there has been considerable interest particularly in the genetic variations of this virus. As a result multiple isolates of HIV have been characterized, their genomes cloned and their nucleotide sequences determined (Myers et al., 1995). Gene expression patterns and phylogenetic relationships have also been established (Sharp et al., 1994). From these studies, it is known that genetic variation is the hallmark of this class of viruses. No two viruses are alike, and even within a single species, HIV is present in a form of 'quasispecies'- a swarm of microvariants which are highly related but genetically distinct from each other (Saag et al., 1988; Meyerhans et al., 1989 and Preston et al., 1988).

## 1.1 Structure of HIV-1

Based on electron microscopic morphology and studies of genomic organization and pathogenic features, HIVs are classified as members of the lentivirus subfamily of retroviruses. Like all retroviruses, HIVs have a cone-shaped core composed of the viral p24 (Gag) protein. Inside this capsid (CA), or nucleoid, are two



identical RNA strands with which the viral RNA-dependent DNA polymerase (Pol), also called the reverse transcriptase (RT) (p66) and the nucleocapsid (NC) protein (P9) are closely associated. The inner portion of the viral membrane is surrounded by a p17 core (Gag) protein that provides the matrix (MA) for the viral structure and is vital for the integrity of the virus (Levy, 1993); Fig.1). The MA is also required for incorporation of the envelope (Env) proteins into mature virion.

The viral surface is covered with characteristic knobs that represent oligomeric structures (tetramers or trimers) of the virally encoded envelope glycoproteins gp120 (SU) and gp41 (TM). The gp120 subunit comprises the extracellular portion of the viral envelope, and the gp41 portion spans the membrane and anchors the glycoprotein complex to the surface of the virion (Fig.1).

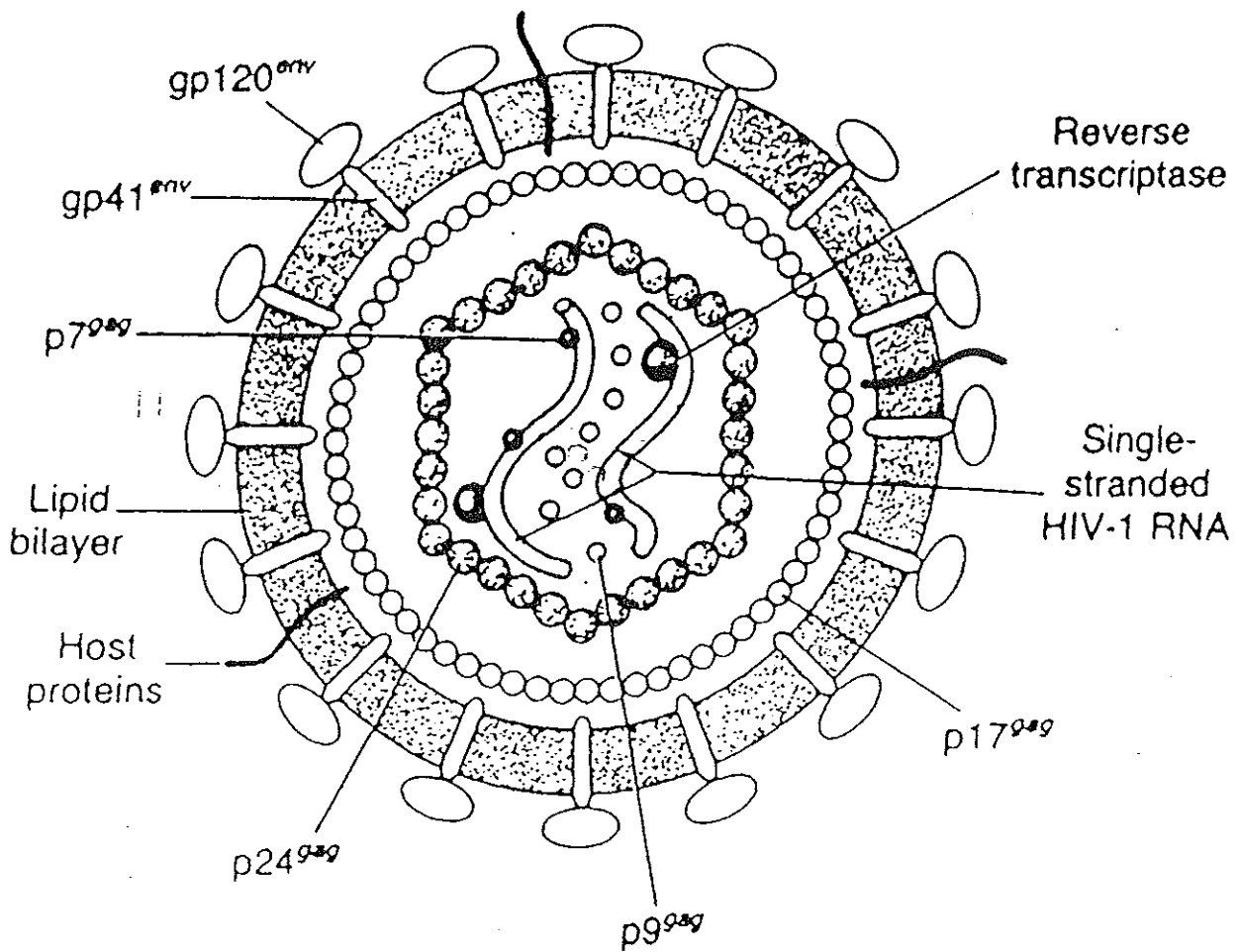


Fig.1. The major structural components of HIV (Source: Constantine et al., 1992)

## 1.2 HIV-1 Genome

The genome of HIV-1 (approximately 10kb) contains nine genes, flanked by long terminal repeats (LTRs) (Fig. 2). The LTRs are non-coding regions and consist of regulatory elements for integration, transcription and polyadenylation of mRNAs. The gag and pol genes code for the core proteins (p24, p17, p9 and p7) and the viral enzymes (reverse transcriptase, protease and integrase), respectively (Muesing et al., 1985). The env gene codes for a 160 kb glycoprotein that is processed into the gp41 transmembrane protein and the gp120 external protein. In addition, there are six small genes: *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (*vpx* in HIV-2). At least three of these genes (*tat*, *rev* and *nef*) are involved in the regulation of viral multiplication (Cullen, 1993).

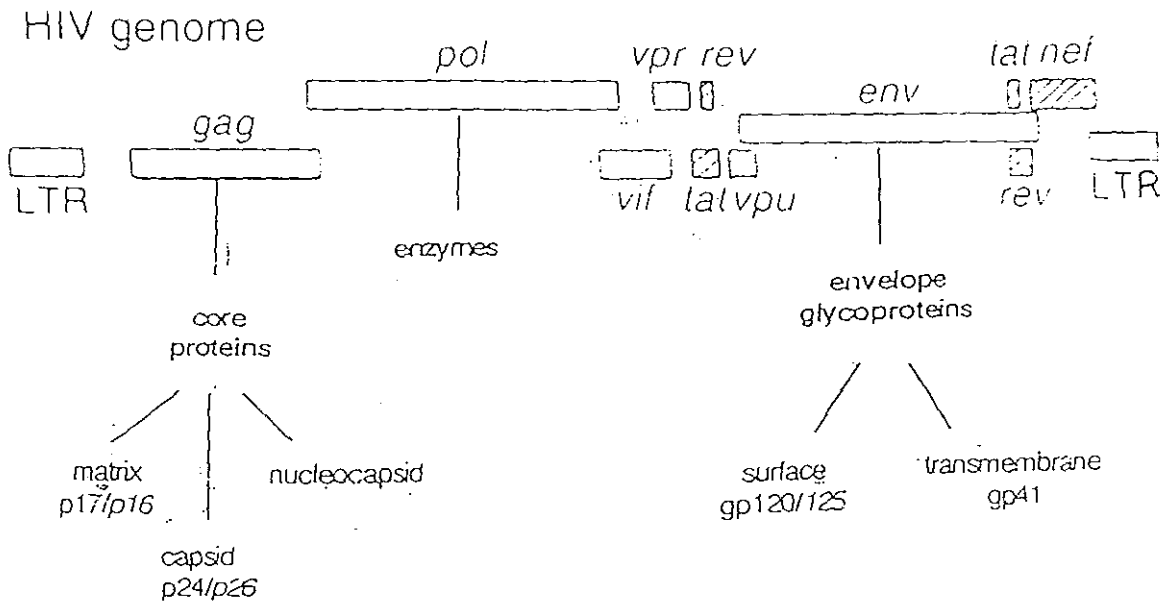


Fig. 2. The HIV-1 genome and encoded protein products. (Source: WHO, 1994).

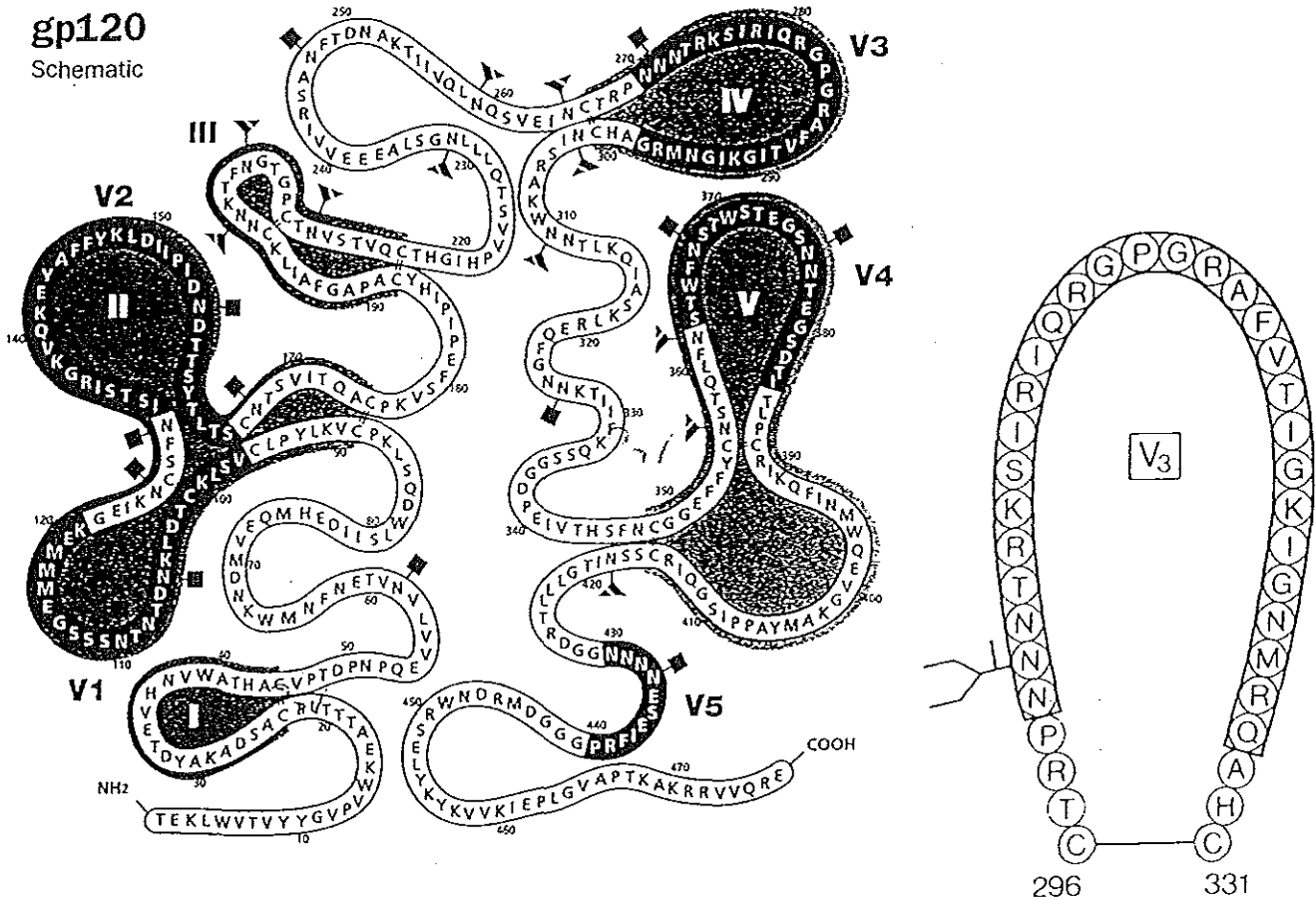
### 1.3 Genetic Variability in HIV-1

HIV (particularly HIV-1) shows extensive genetic variation at various levels (WHO, 1994). HIV-1 viral isolates from North America, Europe and Central Africa, have only 50% nucleotide sequence homology with HIV-2, a related retrovirus common in West Africa. HIV-1 Isolates collected in geographically distinct locations may be divergent by upto 20-30%. Variability in the *env* gene of HIV-1 isolates from different individuals in a single region is in the range of 6-19%, although differences higher than 30% have been noted (WHO, 1994). People infected with a virus from a common source, like a group of hemophiliacs infected through the same HIV-1-contaminated batch of Factor VIII, harbor viruses that are more related to each other (2-5% differences) than to viruses in the general population. There can also be inpatient variation (9%), observed as change over time and as closely related variants within one isolate.

Genetic variation of HIV-1 can alter structure, function and immunogenicity of major viral gene products particularly of the envelope genes, in biologically important ways (Sharp et al., 1994). The *env* gene which is approximately 2500 bp in length, is the most variable (Gao et al., 1994), while the *gag* and *pol* genes are relatively conserved. Variation in *env* occurs with higher frequency in certain regions. On the basis of this clustered variation, gp120 is divided into alternatively conserved (C) and variable (V) domains (Cullen, 1993; Fig.3). These are further

divided into five constant (C1-C5) and variable (V1-V5) regions. Within the third variable domain of gp120, a cluster of amino acid residues flanked by cysteine (the V3 loop) shows high variability and constitutes a major immunodominant epitope (La Rosa et al., 1990), that serves as a major target for specific neutralizing antibodies (Wolfs et al., 1991). This segment of the envelope, which forms a disulfide loop between cysteine residues at gp120 amino acids 296 and 330, is called the principal neutralization domain (PND) (LaRosa et al., 1990). Sequence variation in this region has also been associated with generation of viral mutants in vitro and in vivo (Cullen, 1993), and results in alteration of several important features of HIV-1 strains such as cell tropism (Hwang et al., 1991), susceptibility to neutralization (Javaherian et al., 1989) and syncytium induction (de Jong et al., 1992). Moreover, the V3 loop is responsible for evoking cellular immune responses and has been shown to play a significant role in inflicting cell damage and cytopathicity (Safrit et al., 1994).

gp120  
Schematic







 Glycylation sites containing high manose-type and/or hybrid type oligosaccharide structures  
 Glycosylation sites containing complex-type oligosaccharide structures  
 Five disulfide-bonded domains are labeled by roman numerals  
 Five hypervariable regions of are darkened and labeled V1-V5

Fig. 3. Organization of HIV-1 gp120 molecule. (Source: de Jong, 1994).

#### 1.4 Genetic Subtypes of HIV-1

Based on phylogenetic analysis of diverse *env* and *gag* gene sequences from diverse viral isolates from many countries, HIV-1 has been classified into several distinct genetic subtypes or clades (Pau et al., 1993; Delwart et al., 1994; Bachmann et al., 1994; Bruce et al., 1994; Delwart et al., 1995a, 1995b, 1995c and 1995d and Cheingsong-Popov et al., 1994). Currently, these genetic subtypes have been placed into two distinct major clusters. One cluster, major (M) group comprises the vast majority of HIV-1 and can be further subdivided into at least ten genetic subtypes designated A to J (Leitner et al., 1995; Kostrikis et al., 1995; Louwagie et al., 1993 and 1995 and Sharp et al., 1994). The second major cluster, which has been discovered only recently, has been termed group O for "genetic outliers", and thus far includes only a small number of viruses from Cameroon and Gabon (Charneau et al., 1994; Gurtler et al., 1994; Loussert-Ajaka et al., 1995 and Vanden-Haesevelde et al., 1994). Both groups are characterized by considerable sequence diversity. For example, *env* amino acid sequence variation within group M ranges from 3 to 23% among members of the same subtype and from 25 to 35% among members of different subtypes (Gao et al., 1996). Group O viruses exhibit similar levels of diversity to that of M, but have not been classified into subtypes because of the small number of representatives characterized (Loussert-Ajaka et al., 1995). The two groups, M and O, differ by more than 50% in their *env* protein sequences (Gurtler et al., 1994;

Louwagie et al., 1993 and Vanden-Haesevelde et al., 1994).

The distribution of subtypes varies by local, with multiple subtypes co-circulating in many areas of the world (Artenstein et al., 1995). HIV-1 of subtype B predominates in the United States, Europe, South America, Thailand and Japan, whereas subtype A and D are mainly found in Central, West and East Africa. Subtype C has been found in Eastern and Southern Africa, as well as in India and China. The other subtypes show a more patchy distribution; subtype E in Central African Republic, Thailand, Japan, India, Cambodia and Vietnam; subtype F in Zaire, Brazil, and Romania; subtypes G and H, in Zaire and Gabon and subtype I in Cyprus (Dumitrescu et al., 1994; Kostrikis et al., 1995; Louwagie et al., 1995; Louwagie et al., 1993 and Delaporte et al., 1996).

In many countries, two or more subtypes coexist. Thus, subtype A, D and G have been found in Uganda (Bruce et al., 1994); subtypes B and E in Thailand (Ou et al., 1992 and 1993); subtypes B and F in Brazil (Sabino et al., 1994); subtypes A, B, E, F, H and group O viruses in Cameroon (Nkengasong et al., 1994); subtypes A, C, D, F, G and group O in Gabon (Delaporte et al., 1996) and subtypes A, C, D, E, G and H in Central African Republic (Murphy et al., 1993). The large variety of circulating subtypes in Central Africa seems to be quite different from what has been found so far in other parts of the world, including other regions of Africa where on a similar number of samples,

circulating strains belong only to a few different HIV-1 subtypes.

The main factor behind the geographic distribution of the different subtypes is thought to be due to viral trafficking linked to human behavior (Myers, 1994). The occurrence of subtype B in homosexuals and drug addicts in Western countries is the consequence of epidemiological factors, such as founder effects. The relatively closed nature of the sexual networks of subtype B-infected homosexuals and drug addicts may have contributed to the apparent compartmentalization and the relatively restricted spread of the B subtype in these countries. A similar founder effect is also found in some other communities. For example, all HIV-infected nursed Romanian children were infected by subtype F (Dumitrescu et al., 1994), heterosexual transmission in Thailand mainly involves subtype E (Ou et al., 1992), and a cluster of subtype G infections has been well documented in Russia (Cheinsong-Popov et al., 1993). By contrast, in the inter-tropical African countries, all studies have shown a high degree of diversity, and a founder effect has never been clearly identified (Louwagie et al., 1993 and Nkengasong et al., 1994).

Each subtype of HIV-1 is transmissible sexually, parenterally and vertically, and each causes clinical disease in infected persons (Artenstein et al., 1995). The distribution of HIV-1 subtypes can be correlated to certain specific transmission routes (Dillner, 1996). For example, in the West, HIV-1 is

contracted primarily through homosexual contact and intravenous drug use and HIV-1B is the predominant subtype in such settings (Dillner, 1996). In South Asia and sub-Saharan Africa, HIV is mainly (90%) transmitted through heterosexual contact (Dillner, 1996) and consists of the subtypes A, C, and E (Ou et al., 1993 and 1992).

The assertion that HIV-1 genetic subtypes have differential cell tropisms (Soto-Ramirez et al., 1996) has now been disproved (Pope et al., 1997 and Dittmar et al., 1997). The thought that HIV-1 strains of subtype E and C are more transmissible between heterosexuals than subtype B because they have an unusual capacity to replicate in Langerhans' cells (LC) (Soto-Ramirez et al., 1996) is no longer accepted since HIV-1 isolates from heterosexually infected patients did not show preferred tropism for LC's compared to homosexually transmitted HIV-1 isolates (Pope et al., 1997). Therefore, no subtype-specific pattern of infection has been shown to exist and the observed patterns of spread may be due to social and epidemiological factors.

Consensus sequences have been constructed for each HIV-1 genetic subtype, by putting at each position the amino acid that most frequently appears there (Kuiken and Korber, 1994) (Fig. 4). The consensus sequences are stored in a central database at Los Alamos (USA) and regularly updated. The sequences are aligned to a consensus based on the most common amino acid in the subtype consensus sequences, which approximates a "global" consensus

(Foley and Korber, 1995). Certainly many V3 loop variants in each of the subtypes are extremely divergent from the consensus sequences (Foley and Korber, 1995). These divergent forms may have very different biological and immunological characteristics from viruses which are similar to the consensus.

SUBTYPE	V3 AMINO ACID SEQUENCE	GEOGRAPHIC DISTRIBUTION
ALL CONSENSUS	CTRP.NNNTRKS...IRI...GPGQ..AFYAT...GDIIG...DIRQAHC	
A CONSENSUS	-----V-----	WEST, CENTRAL AND EAST AFRICA
B CONSENSUS	-----H-----R-----T-----E-----	AMERICAS, EUROPE, THAILAND AND JAPAN
C CONSENSUS	-----T-----	EASTERN, SOUTHERN, AND CENTRAL AFRICA, CHINA AND INDIA
D CONSENSUS	----Y---QR---TH-----L-T---.R-----	CENTRAL, EAST AND SOUTHERN AFRICA
E CONSENSUS	----S---T---T-----V--R-----K-Y-	CENTRAL AFRICA, THAILAND, JAPAN, CAMBODIA AND VIETNAM
F CONSENSUS	-----HL-----K--	RUMANIA, BRAZIL AND ZAIRE
G CONSENSUS	-----TF-----	WEST AFRICA
H CONSENSUS	-----R---MS-----R--GQ-F-----Y-	WEST AFRICA
O CONSENSUS	-E---Q-QVQQ.---YT---MA--.W-SM---.STTTSTRS--.Y-	GABON AND CAMEROON

Fig. 4. HIV-1 Subtype Consensus Sequences and their Geographic Distribution. This V3 region alignment shows amino acid consensus sequence generated for each of the nine subtypes (consensus sequences for subtype I and J are not shown). The nine subtype consensus sequences indicate the most common amino acid found in each position among the sequences associated with each subtype. The sequences are aligned to a consensus based on the most common amino acid in the subtype consensus sequences, which approximates a "global" consensus. As is the convention in this compendium, a dash(-) indicates concurrence with the top sequence in the alignment; a period (.) indicates a deletion. (Source: Foley and Korber, 1995).

## 1.5 Causes of Genetic Variability in HIV-1

The considerable variation of the different genetic subtypes noted above are thought to have arisen due to different factors such as mutation, immune selection and recombination (Bachmann et al., 1994).

### 1.5.1 Mutation

HIV-1 has a high rate of mutation (Coffin, 1986). In the plasma, the virus appears to turnover rapidly with a virion half-life of approximately 6h and an estimated  $10^9$  virus particles generated daily (Ho et al., 1995). With the production of  $10^9$  virions daily and a genome size of  $10^4$  nucleotides, virtually all possible mutations are generated daily. This high rate of mutagenesis is shared by other retroviruses (Preston et al., 1988) and presumably originates in mechanisms unique to the retroviral life cycle. Replication of retroviral genomes is proceeded by a series of enzymatic reactions involving virus-encoded reverse-transcriptase (RT) and integrase, as well as host cell encoded DNA polymerases and RNA polymerase II (Preston et al., 1988). The viral RT polymerizes deoxyribonucleotides by using viral RNA as a template and also acts as a DNA polymerase in converting the resulting minus strand DNA into double-stranded DNA (Preston et al., 1988). During the reverse transcription process which has no proof-reading mechanisms, it introduces nucleotide sequence changes at a rate of approximately one

substitution per genome per replication cycle (Preston et al., 1988 and Roberts et al., 1988). Therefore, because of its high mutation rate and strong selective pressures by the immune system for the emergence of phenotypic variants, the HIV-1 *env* gene evolves rapidly through nonsynonymous nucleotide substitutions and short-in-frame insertions and deletions (Delwart et al., 1995a). Such mutations together with silent, synonymous nucleotide substitutions lead to the formation of genetic variants in HIV-1 viruses (Delwart et al., 1995a). This reservoir of genetic variants or 'quasispecies' enables the virus to adapt rapidly to its changing environment, for example by infecting 'new' target cells or replicating in the presence of drug/immune response.

### *1.5.2 Immune Selection*

Evidence exists that the immune response to HIV-1 results in the continuous selection of new viral variants (Michael et al., 1996). Soon after primary infection, a genetically homogeneous virus population is detected in peripheral blood mononuclear cells (PBMC) (Delwart et al., 1995a), although variants might hide in the lymph node. At variable times after infection, genetic changes lead to the formation of a diversified viral population often referred to as quasispecies (Saag et al., 1988; Meyerhans et al., 1989 and Preston, et al., 1988). Over time HIV-1 quasispecies also change, as one set of variants is replaced by another, presumably a more fitter set of variants

(Delwart et al., 1995a). This happens because the immune system changes as it tries to suppress the virus from spreading. As a result, the observed distribution of genetic forms early on in a quasispecies can undergo continuous change under the influence of a large number of factors (Kuiken and Korber, 1994). For example, at a microscopic level, the immune system of a specific host may be critical, and this factor may be altered as the disease progresses (Kuiken and Korber, 1994). At the macroscopic level, the sociological factors such as risk behaviors or the genetic backgrounds of different populations may influence the distribution of variants in the quasispecies (Kuiken and Korber, 1994).

### *1.5.3 Recombination*

Genetic recombination is also an important contributory factor to the rapid generation of sequence diversity seen in HIV-1 over the course of infection within an individual (Sabino et al., 1994) and also over the course of the epidemic, particularly in geographic areas where multiple subtypes of HIV-1 co-circulate (Artenstein et al. 1995). A retrospective analysis has established that a significant fraction, perhaps more than 10%, of reported HIV-1 strains represented mosaic or recombinant forms, bearing interspersed segments of genetic information from two different genetic subtypes (Robertson et al., 1995). This recombination which occurs between viruses from different subtypes could pose a further challenge to the already complex

task of developing vaccines effective against the multiple subtypes of this rapidly evolving pathogen. In addition, recombination between viruses of distinct strains would suggest that serial HIV-1 infections in individuals can occur, which in itself would be an impediment to vaccine development efforts (Sabino et al., 1994).

### *1.6 Functional Significance of HIV-1 Variability*

The high level of genetic variation in HIV-1 has led to extensive biologic and serologic heterogeneity of this virus (Levy, 1993). Biologically, it has led to differences in certain important functions such as cellular tropism, replication kinetics, level of virus production, cytopathicity, ability to form syncytium, latency and inducibility. Serologically, it has caused variations in the susceptibility of various HIV-1 strains to serum neutralization (Levy, 1993).

The significance of viral heterogeneity in disease pathogenesis is not yet fully understood (Fauci and Rosenberg, 1994). Differences in the degree of in vitro cytopathicity and in the number of in vitro cell types that can be infected have also been observed in isolates within a single individual (Spira et al., 1995). The isolation of HIV-1 variants with increased in vitro cytopathicity, increasing replication rates, syncytia-forming capacity, and ability to replicate in wider range of cells has been associated with progression to AIDS (Fauci and

Rosenberg, 1994).

Genetic variation may enable the virus to escape immune surveillance (Albert et al., 1993). The human immune system is confronted by the patchwork of conserved and variable domains in the envelope glycoprotein of HIV-1 (Cullen, 1993). Rapid variation in the amino acid sequence in the variable regions permits the virus to evade neutralization by preexisting antibodies (Coffin, 1986). In addition, the N-linked carbohydrate side chains present in this region, in some way serve as "umbrellas" to prevent the more conserved regions from being available for neutralizing or cell-mediated response (Coffin, 1986). In so doing, the virus will be able to "outrun" the immune response by rapidly diverging into a population of related but distinct species which could then generate additional variation by recombination with one another (Sabino et al., 1994). Even the more conserved *gag* polyprotein gene, encoding matrix, capsid and nucleocapsid proteins internal to the virion, exhibits considerable interisolate diversity and is the target of both cellular and a humoral immune response (Louwagie et al., 1993).

The extensive genetic variability of HIV-1 poses a formidable challenge to the development of broadly effective HIV vaccines (Haynes, 1996). Subtype distribution varies from country to country and changes over time. This will even continue to change as persons travel within and between countries (Kalish et al., 1995). Therefore, as global surveillance continues, it is

impossible to predict how many subtypes of HIV-1 may ultimately be identified. Even group O viruses, which are as different from each other as are viral subtypes A-I, may be further subdivided into another major cluster of HIV-1.

Because of this extreme degree of genetic divergence, HIV vaccine formulation may require immunogenic proteins from more than one viral subtype (vaccine cocktails) to elicit a broad protective immune response against multiple subtypes of viruses (Kalish et al., 1995). To include proteins from multiple strains of HIV-1, vaccines may need to be tailored to the local strains circulating within a country or geographic region. In line with this, the Global Program on AIDS of the World Health Organization (GPA/WHO) has established the "Network for HIV Isolation and Characterization" (WHO-NHIC), for monitoring HIV variability globally (Osmanov et al., 1994).

In addition, the high genetic variation of HIV has significant implications for the sensitivity and specificity of diagnostic methods. The isolation of HIV-1 in 1983 (Barre-Sinoussi et al., 1983) and the development of serological tests soon thereafter were major breakthroughs. However, the subsequent identification of HIV-2 required significant modification of diagnostic tests. The identification of the highly divergent genetically group O strains of HIV-1 required another modification of some diagnostic tests, particularly those utilizing synthetic peptides and recombinant proteins as

antigens. At the same time, the techniques based on whole virus lysates (Western blot) still are capable of reliably detecting all HIV-1 variants. The newer generation of laboratory techniques for HIV detection have been modified to include the group O specific antigens and thus improving the sensitivity and specificity of currently available HIV diagnostic techniques (Nkengasong et al., 1994 and Gurtler et al., 1994).

This experience with group O HIV-1 has heightened awareness of the possible problems that may arise in the case of emergence of new highly divergent genetic variants, which may render the existing diagnostic tools less efficient and undermine their value for public health. It is therefore important to monitor the sensitivity and specificity of all current and future diagnostic kits.

### *1.7 HIV-1 Subtyping Techniques*

With regard to HIV-1 subtyping, various techniques, amongst which the most important are: peptide ELISA (Wasi et al., 1995), heteroduplex mobility assay (Delwart et al., 1995b) and PCR aided DNA sequencing (Simmonds et al., 1990) can be used. An algorithm which uses these techniques in the above order would generate reliable information on HIV-1 subtypes (Wasi et al., 1995).

Preliminary Virus typing can be performed serologically by using synthetic linear V3 peptides which are adsorbed passively

onto an ELISA microtiter plate, and then bound to specific antibody that can be detected by a secondary antibody -enzyme conjugate (peptide ELISA) (Cheingsong-Popov et al., 1992). The synthesized peptides represent the consensus sequences of the V3 loop from various subtypes and are antigenic for HIV-1 seropositive human sera. Peptide serotyping is a simple, rapid and affordable tool suitable for large-scale screening studies of the HIV-1 subtype distribution (Cheingsong-Popov et al., 1992). As a result, it has been applied as a primary screening tool to direct the investigation of HIV-1 diversity in many countries of the world (Cheingsong-Popov et al., 1993 and 1994). However, one disadvantage of peptide serology is that some cross-reactivity is reflected in the genetic relatedness of subtypes (e.g. A and C are highly cross-reactive) (Cheingsong et al., 1994).

The DNA heteroduplex mobility assay (HMA) has been used by several researchers to determine evolutionary relationships of HIV-1 *env* genes from geographically diverse HIV-1 isolates (Delwart et al., 1994; Wasi et al., 1995; Bachmann et al., 1994 and Bruce et al., 1994). HMA is based on the principle of migration differences of hetero- and homo-duplexes in polyacrylamide gels (Delwart et al., 1995b). DNA sequences from a set of reference isolates of known genetic identity can be used to form heteroduplexes with samples of unknown sequence identity. A 1.2kb *gpl20* fragment encompassing the V1-V5 region of the *env* gene is most commonly analyzed, although other genomic regions can also be used. Viral gene fragments are amplified by nested

PCR using DNA templates from virus culture or primary uncultured material. The same size fragments are also amplified from a series of plasmids containing HIV-1 env genes from different subtypes used as references. The electrophoretic mobility of heteroduplexes formed between the unknown sample and the reference sequences is then determined on a 5% polyacrylamide gel. Homoduplexes migrate fast and run to the bottom of the gel, while heteroduplexes migrate more slowly, with their degree of retardation being dependent on the extent of sequence mismatch between the two strands. As a result, the mobility of heteroduplexes formed between sufficiently divergent molecules is reduced relative to that of the fully complementary homoduplex molecules (Delwart et al., 1995b). These mismatches are thought to result in "bubbles" in the heteroduplex which retard the mobility of the fragment through the polyacrylamide matrix.

When compared to peptide ELISA, HMA is in general more reliable because of higher sensitivity and specificity and allows for a more definitive determination of HIV-1 subtypes. However, this method is expensive and has high demand on the sample quality (Wasi et al., 1995). Therefore, the combination of the two techniques, that is, an initial serological screening by peptide ELISA and subsequent HMA analysis of non-typable or dually reactive specimens could be a strategy of choice.

DNA sequencing can be used for the precise subtyping of HIV-1. In general, this technique is more time-consuming and requires

elaborate techniques such as radioactive material or fluorescent sequencing devices not readily available or too costly for many laboratories. As a result, although DNA sequencing is the generally accepted gold standard for subtyping, it can not be applied to high numbers of samples and certainly there are technical restrictions in Third World countries (WHO, 1994).

### *1.8 HIV-1 Situation in Ethiopia*

In Ethiopia, since the first report of HIV-1 positive sera in 1984 (Tsega et al., 1988) and the first AIDS cases in 1986 (Ayehunie et al., 1987), the epidemic has spread to reach prevalences in 1997 of 14-20% in urban pregnant women, 47-59% in commercial sex workers (CSW) (Ethio-Netherlands AIDS Research Project (ENARP), sentinel survey 1997, personal communication) and 7% in blood donors (Ethiopian Red Cross Society, National Blood Transfusion Service (ERCS-NBTS), 1994 report, personal communication). The Ethiopian HIV-1 epidemic has been shown to be dominated by subtype C (Ayehunie et al., 1990; 1991 and 1993; Johansson et al., 1995; Sherefa et al., 1994a, b and 1997 and Salminen et al., 1996 and Abebe et al., 1997). The presence of subtype C in Addis Ababa was revealed first in 1991, by preliminary DNA sequencing of *gag* and *env* on HIV-1 isolates of ARC/AIDS patients in Addis Ababa (Ayehunie et al., 1991 and Ayehunie et al., 1993). Other studies done by using peptide ELISA (Sherefa et al., 1994a) and DNA sequencing (Abebe et al., 1997) further confirmed the presence of subtype C as the major

HIV-1 subtype in Addis Ababa. Recently, the sequencing of 94 viral isolates collected between 1989 and 1995 from various risk groups in Addis Ababa showed only one HIV-1 isolate to be of subtype A (pregnant woman, 1995) (Abebe et al., 1997). Furthermore, within the above 93 HIV-1 subtype C isolates, two different subclusters were detected, suggesting the possibility of two independent introductions of HIV-1 C subtype viruses in Ethiopia (Abebe et al., 1997). A full length Ethiopian HIV-1 subtype C sequence of a 1986 isolate was published in 1996, revealing the existence of three potential transcription factor binding sites (Salminen et al., 1996). Finally, another full length Ethiopian HIV-1 sequence was recently published, documenting the first evidence of a subtype A/C recombinant in a sample from Addis Ababa, collected from a 34 year old male in October 1991 (Sherefa et al., 1998).

Recently, a study amongst French army recruits heterosexually infected with HIV-1 in Djibouti demonstrated the presence of a much wider range of subtypes: A, B, C, D and E (Lasky et al., 1997). The fact that Djibouti is a neighboring country of Ethiopia, its harbor being part of a major trade route to Addis Ababa and that Ethiopian commercial sex workers are reportedly working in Djibouti, points to the possibility of the influx of new HIV-1 subtypes from this area into Ethiopia.

In this context, this study aims at assessing whether, and to what extent, non-C HIV-1 subtypes are being introduced into

Addis Ababa in 1997. It also aims at setting up and validating techniques for HIV-1 subtyping in an Ethiopian context. Knowledge about the various subtypes present in Ethiopia might be important for future vaccine development and implementation; for the study of the epidemiology of transmission and for the sensitivity of diagnostic assays.

## 2. MATERIALS AND METHODS

### 2.1 STUDY POPULATION

The study included 150 commercial sex workers (CSW) from Tekle-Haimanot (n=75) and Kazanchis (n=75) Health Centers in Addis Ababa. Subjects were enrolled in order of arrival and HIV-1 tests were performed in an unlinked anonymous way, according to WHO guidelines (WHO, 1989). Since the samples were collected as part of the existing collaborative structure between the Ethio-Netherlands AIDS Research Project (ENARP) and Tekle-Haimanot and Kazanchis Health Centers, syphilis serology was performed by TPPA (Serodia-TPPA, Fujirebio, Japan) and RPR (BioMerieux, France) assays, according to the manufacturer's instructions. Syphilis results were returned to the Health Centers and penicillin treatment was provided to the study participants for free.

### 2.2 SAMPLE COLLECTION

Whole blood samples (10 ml) were collected from each individual in EDTA vacutainers by laboratory technicians from Tekle-Haimanot and Kazanchis Health Centers. Age and number of years spent as a CSW were recorded on coded forms at the time of blood collection. The blood samples and the forms were

transported to ENARP's laboratory.

## 2.3 PROCESSING AND LABORATORY INVESTIGATION OF SAMPLES

### 2.3.1 Plasma Isolation and Selection of HIV

#### *Positive Samples by HIV-SPOT.*

Plasma was isolated by centrifuging blood samples in EDTA vacutainer tubes at 300g (1640 rpm) for 10 minutes with brake. The plasma was then transferred with a sterile pipette into a labeled 15 ml tube. Earles' Heparin New Born Calf Serum (EHN) (475ml Earles', 25ml New Born Calf Serum (NBCS), 2ml heparin (20ul/ml) and 1ml penicillin/streptomycin (100ul/ml;100ug/ml), Life Technologies, Scotland) medium was added into the vacutainer tubes, mixed carefully and the tube was stored in a laminar flow at room temperature until peripheral blood mononuclear cells (PBMC) were isolated (see below).

One drop of each plasma sample was taken and HIV-SPOT test (Genelabs Diagnostics, Singapore) was performed as described by Nkengasong et al., 1992. The test involves trapping of antibodies to HIV-1 and/or HIV-2 by the capture reagents which are adsorbed to a porous membrane. The capture reagent encompasses a recombinant partial protein of HIV-1, corresponding to a region overlapping the junction between the gp120 and gp41 fragment of the envelope protein. A highly purified peptide

which corresponds to a region of the envelope transmembrane protein of HIV-2 is also immobilized to the membrane. A porous plastic is used as a solid phase support where the antigen is coated. According to the procedure, 3 drops of reconstituted liquid buffer (Tween 20, bovine serum albumin and heat treated gold serum) was added and allowed to soak. This was followed by the addition of 1 drop of undiluted sample and the strong reactive control (Inactivated lyophilized human serum containing antibodies to HIV-1 antigens), and non-reactive control (normal human serum). Then 2 drops of reconstituted liquid buffer and 2 drops of wash buffer (deionized water) were added. Finally, after addition of 2 drops of reconstituted gold conjugate (protein A-gold reagent), the devices were washed by using 3 drops of wash buffer (deionized water) solution. Results were read within 10 minutes visually. HIV-1 positivity was indicated by a distinct red spot on the membrane and negativity by a clear membrane or an overall pink color.

The 15 ml plasma tubes of selected samples (HIV-SPOT positive) were centrifuged again for 10 minutes at 2500 rpm at room temperature. Using sterile pipette the cell-free plasma was transferred from the 15ml tube to three freezing vials. The freezing vials were then immediately stored at -80°C.

### 2.3.2 PBMC Isolation for DNA

PBMC were isolated by Ficoll-Hypaque (Pharmacia Biotech, Sweden) density gradient centrifugation based on the procedure specified by the manufacturer. In brief, the blood samples with EHN medium were transferred from the EDTA vacutainers into sterile 50 ml tubes. The vacutainer tubes were rinsed with 5 ml of EHN medium and this was pooled into the corresponding 50 ml tube. EHN medium was added to the 50 ml tube upto 20 ml level and was then mixed carefully using a pasteur pipette. The mixture was then carefully pipetted on top of corresponding tubes containing 12.5 ml Ficoll and these tubes were centrifuged at 2200 rpm for 15 minutes at room temperature without brake. After aspirating the supernatant until 1 cm above the Ficoll layer, the cell band on top of the Ficoll was transferred using a sterile pipette to new 50 ml corresponding tubes. The harvested cells were washed by adding EHN medium and centrifuged at 1500 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet loosened by gently tapping. To this PBMC pellet, 0.9 ml IF20 medium (200 ml ISCOVES, 50 ml Foetal Calf Serum (FCS) and 0.5ml penicillin/streptomycin) was added.

The total number of PBMC (in 1 ml) was then automatically counted using a Coulter Counter (Coulter Counter, Electronics Ltd, NY). The volume of sample containing exactly  $1 \times 10^6$  PBMC was calculated and this amount pipetted into a 1.5 ml Eppendorf tube containing 750 ul L6 lysis buffer (120g Guanidine/100ml L1 buffer

(12.1g Tris/900 ml H<sub>2</sub>O and 8.1 ml 37% HCl PH=6.4), 0.5M EDTA PH=8.0 and 2.4ml Triton X-100). This was stored at -20°C. The remaining PBMC were frozen in liquid nitrogen in 2 separate tubes.

### 2.3.3 HIV Serology

All HIV-SPOT positive plasma were re-screened qualitatively for levels of antibodies to HIV antigen by the ELISA (Vironostika<sup>R</sup> HIV Uni-Form II, Organon Teknika, The Netherlands). The kit has ninety six well microtiter plates with the required conjugate, substrate and washing buffer. Each micro-ELISA well contains horseradish peroxidase (HRP)-labeled conjugate sphere of mixture: recombinant HIV-1 p24, viral HIV-1 gp160 and synthetic HIV-2 env peptides.

To each well 100 ul specimen diluent (stabilizing protein and detergent) was dispensed using a multichannel pipette, which dissolved the conjugate spheres. Then, 50 ul test samples and appropriate controls containing anti-HIV-1 and/or anti-HIV-2 monoclonal antibodies were added. The plates were covered with plate sealer and incubated in a water bath at 37°C for an hour. After incubation the wells were washed 6 times with wash phosphate buffer by an automatic ELISA washer (Washer 400, Microwell System, Organon Teknika, the Netherlands). Following the wash procedure 100 ul Tetramethylbenzidine (TMB) substrate was pipetted into each well and incubated at room temperature

(18-25°C) for 30 minutes. Color which turns yellow when the reaction is stopped by adding 100 ul of 1N sulfuric acid, develops for positive specimens. The plates were read at 450 nm by an ELISA reader (Reader 230, Microwell System, Organon Teknika, The Netherlands).

Validity of the test procedure was checked according to the manufacturers instructions. Cut-off value was determined and results were interpreted based on the cut-off value (mean + 0.100). A test sample was considered reactive when sample absorbance reading was found to be greater than or equal to the cut-off value, and non-reactive if sample absorbance is less than the cut-off value (Organon Teknika, The Netherlands). A non-reactive result indicates that the sample tested contains neither anti-HIV-1 nor anti-HIV-2 antibodies or contains anti-HIV-1 and/or anti-HIV-2 antibodies below the detectable limits of Vironostika HIV Uni-Form II assay. A reactive result means that the samples tested either contain anti-HIV-1 and/or anti-HIV-2 antibodies or contain nonspecifically reacting factor(s).

The discrepant HIV-SPOT - ELISA results were confirmed by a Western Blot Assay (HIV BLOT 2.2, Genelabs Diagnostics, Singapore). This assay contains nitrocellulose strips blotted with HIV-1 viral lysate and specific HIV-2 envelope peptide and the required conjugate, substrate, blotting powder, stock and washing buffer concentrate and incubation trays with 9 wells each.

In brief, the required number of nitrocellulose strips (including strips for controls-strong reactive, weak reactive and non-reactive) were removed from the tube using a forceps and placed numbered side up into separate wells of a special tray and incubated with 2ml of diluted wash buffer (Tris with Tween-20) for at least 5 minutes at room temperature on a platform shaker (STUART Scientific, UK). After the buffer was aspirated, 2ml of blotting buffer (5%, non-fat dry milk diluted with stock buffer) was added to each well followed by 20 ul of each patient's plasma and controls to the appropriate wells. The tray was covered with the cover provided and incubated overnight (16-20 hours) at room temperature on the platform shaker. The next day, the tray was uncovered carefully to avoid splashing or mixing of samples and the mixture was aspirated from the wells. Each strip was washed 3 times with 2ml of diluted wash buffer, allowing 5 minutes soak on the platform between each wash. Next, 2ml of working conjugate solution (goat anti-human IgG conjugated with alkaline phosphatase) was added to each well and incubated for 30 minutes on the rocking platform. After aspirating and washing the conjugate as before, 2ml of substrate solution (5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT)) was added to each well and incubated again for 10-15 minutes on the rocking platform. The substrate was aspirated and rinsed several times with reagent grade water to stop the reaction. Using forceps the strips were gently removed onto paper towels and dried. The strips were then mounted on worksheet (non-absorbent white paper) and the results were interpreted according to the

instruction given in the manual. That is, the presence or absence of antibodies to HIV-1 in a sample by the HIV BLOT 2.2 assay is determined by comparing each nitrocellulose strip to the assay control strips. The strong reactive control strips should show all relevant molecular weight bands as shown in the figure given in the HIV BLOT 2.2 manual. The bands are p17, p24, p31, gp41, p51, p55, p66 and gp120/gp160. Other bands associated with core antigens (i.e. p39, p42) may also be visible. The plasma control and HIV-2 specific bands should also be visible. The weak reactive control strips provide a measure of the sensitivity of the kit. Weak bands at p24 and/or gp41 and gp120/160 should appear. Some additional weak bands may or may not be present. The plasma control band should also be visible. The non-reactive control strips on the other hand, should show no specific bands of HIV-1 and HIV-2, though the band for the plasma should be visible. After the molecular weight of bands on each test strip were identified using the strong and/or weak reactive control strips as a guide, interpretation of the results was performed using the American Red Cross criteria.

For all anti-HIV-1 positive samples, peptide ELISA was performed. Four peptides (A-D) derived from the V3-loop region of HIV-1, from the WHO EVA programme, Department of Virology, Academic Medical Center (AMC), Amsterdam, The Netherlands were used as antigens (Fig. 5). A fifth peptide was a tailor-made Ethiopian subtype C peptide, based on previous sequencing data (Abebe et al., 1997). Normal human serum, skimmed milk, H70 (sera

reactive with African samples) and VDH (sera reactive with B samples) (EVA program, Department of Virology, Academic Medical Center (AMC), Amsterdam, The Netherlands) were used as controls.

#### Subtype

A	K S V H I G P G Q A F Y A T
B	K S I H I G P G R A F Y T T
C (cons)	R K S I R I G P G Q T F Y A T
C (Eth.)	R K S I R M G P G Q T F Y A T
D	R Q R T H I G P G Q A L Y T T

Fig.5. The V3 amino acid sequences of the synthetic peptides used in the assay.

The peptide ELISA was performed by first coating 96-well plate(s) (Nunc-Immuno Plate, Maxisorp™ Surface) with 100 ul of 100 ng of peptide(s) (i.e. peptide was diluted from stock solution (1 mg/ml) in sterile 1X phosphate-buffered saline (PBS) (410g NaCl, 75.75g Na<sub>2</sub>HPO<sub>4</sub>, 13.2g NaH<sub>2</sub>PO<sub>4</sub>.H<sub>2</sub>O/L PH=7.4) overnight at room temperature. The next day the plates were washed by an ELISA washer (Washer 400, Microwell System, Organon Teknika, The Netherlands) 6 times with wash buffer (phosphate buffered saline PH 7.4 containing 0.1% Tween-20), by turning the plates 180 degrees after each wash. The plates were dried by tapping on tissue paper. Non-specific binding sites were blocked with 150 ul/well of blocking buffer (PBS containing 7% skimmed milk and

0.3% Tween-20), by incubating for 5 hours at 37°C. The plates were then emptied and dried by tapping gently on tissue paper. Next, after each plasma sample was diluted 1:100 with blocking buffer (PBS containing 7% skimmed milk and 0.3% Tween-20), 100 ul of this diluted specimen was dispensed in the corresponding well (each sample was dispensed in duplicates) and incubated for 1 hour at 37°C. The plates were washed again in a similar manner except now they were washed eight times. Bound antibodies were detected with goat anti-human horseradish peroxidase labelled conjugate (Kirkegard and Perry Lab. Inc.) diluted 1:10,000 in 7% skimmed milk. The conjugate (100 ul/well) was left to react with the bound antibody for 1 hour at 37°C. After washing twelve times with wash buffer, the color was developed for 10 minutes using ortho-phenylenediamine dihydrochloride (OPD) substrate (Abott Laboratories) (100 ul/ well). The reaction was stopped after 4 minutes by adding 100 ul 1N H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) was measured at 450nm within 15 minutes by using ELISA plate reader (Reader 230, Microwell System, Organon Teknika, The Netherlands).

The cut-off value for Ethiopian peptide ELISA assay was previously determined (Abebe, personal communication) as [(average + 4 times standard deviation = 0.90)]. Accordingly, OD readings of 0.9 and above is interpreted as reactive for that peptide(s). Those with OD readings between 0.7 and 0.9 are considered as indeterminate. On the other hand, if OD readings are below 0.7, the sample is considered non-reactive for the pertinent peptide(s).

### 2.3.4 Heteroduplex Mobility Assay (HMA)

#### a) DNA Isolation

DNA was isolated according to Boom et al. (1990). Accordingly, Eppendorf tubes containing L6 DNA ( $1 \times 10^6$  PBMCs) were thawed and 750  $\mu$ l of isopropanol was added. After mixing the tubes were centrifuged at full speed (14000 rpm) in Eppendorf centrifuge for 15 minutes at room temperature. The supernatant was aspirated and the DNA pellet was washed twice with 70% ethanol and centrifuged at full speed for 5 minutes. The supernatants were decanted and the pellets were air dried by placing inverted Eppendorf tubes on tissue paper. To dissolve DNA pellets, 50  $\mu$ l of sterile demineralized water was added and stored overnight at 4°C.

#### b) Polymerase Chain Reaction (PCR)

The PBMC DNA (5  $\mu$ l, equivalent to 100,000 cells) was first amplified in a 50  $\mu$ l reaction mixture containing 5  $\mu$ l of 10X MgCl<sub>2</sub> free buffer (Promega, U.S.A.), 6  $\mu$ l of 25mM MgCl<sub>2</sub> (3mM final) (Promega, U.S.A.), 27.5  $\mu$ l of demineralized H<sub>2</sub>O, 2  $\mu$ l 10x deoxynucleotide triphosphate (dNTP) mix (0.2mM final), 2  $\mu$ l (10pmol) of primers ED3 (5'-TTAGGCATCTCCTATGGCAGGAAGAAGCGG-3' (5537-5566 nt) and ED14 (5'-TCTTGCCTGGAGCTGCTTGATGCCCCAGAC-3' (7538-7509 nt) or as an alternative, ED5 (5'-

ATGGGATCAAAGCCTAAAGCCATGTG-3' (6134-6159 nt) and ED12 (5'-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG-3' (7388-7359 nt) primers (NIH AIDS Research and Reference Reagent Program, U.S.A.) and 0.5 ul of Taq polymerase (2.5 U) (Promega, U.S.A.) were used. The alternative primers were used whenever the ED3/ED14 primer did not result in amplification. The reaction was cycled 36 times (94°C, 55°C and 72°C for 1 minute (5 cycles), 94°C for 15 seconds, 55°C for 45 seconds and 72°C for 1 minute (30 cycles) and 72°C for 5 minutes (1 cycle) using a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, U.S.A.). The primers ED3/ED14 amplify approximately a 2 Kb product spanning from the first exon of rev to the gp41 coding region of env while the ED5/ED12 primer amplifies approximately a 1.25 Kb product spanning the V1-V5 coding region of gp120.

2ul of the first round PCR product were reamplified in 100 ul reaction mixture having the same reagents as before, with the primers ED5/ED12; ED31/ED33 or ES7/ES8. The reaction was also cycled in the same manner as before except that the 5 cycles were now reduced to 3 cycles and the 30 cycles were increased to 32 cycles. The reaction mixture alone was included as a negative control in each PCR reaction, while 1ul of subtype C reference plasmid DNA (10ng/ul) was included in the second round reaction as a positive control. The ED31 (5'-CCTCAGCCATTACACAGGCCTGTCCAAAG-3' (6816-6844 nt) and ED33 (5'-TTACAGTAGAAAAATTCCCCTC-3' (7380-7359 nt) primers amplify a 0.5 Kb product spanning from C2-C3 region while the ES7 (5'-tgtaaaacgacggccagtCTGTTAAATGGCAGTCTAGC (7001-7020 nt) and ES8

(5'-caggaaacagctatgaccCACTTCTTCTCCAATTGTCCCTCA (7647-7667 nt)  
primers amplify a 0.7Kb spanning from V3-V5 region.

One ul of reference plasmid DNA (10ng/ul) was also amplified using the second round primers only. The reference subtypes used were: for HIV-1 subtype A: A1 (RW20, Rwanda), A2 (IC144, Ivory Coast), A3 (SF170, Rwanda); for HIV-1 subtype B: B1 (BR20, Brazil); for HIV-1 subtype C: C1 (MA959, Malawi), C2 (ZM18, Zambia), C3 (IN868, India), C4 (BR25, Brazil); for HIV-1 subtype D: D1 (UG21, Uganda), D3 (UG46, Uganda) and for HIV-1 subtype E: E1 (TH22, Thailand).

To see if the PCR was successful, 5 ul of the second round PCR product and a DNA length marker (50ng/ ul of 100 bp DNA marker, Lambda marker or *Hind* III *Eco*RI marker), were loaded on a 1% agarose gels (agarose, tris-acetate-EDTA buffer (TAE) (40mM Tris, 33mM acetic acid and 2mM EDTA) and 3 ul of Ethidium Bromide (0.5ug/ml) with 5 ul of loading dye (40% sucrose and 0.015 % Bromophenol Blue). After the gel was electrophoresed at 100 mV for 1 hour in TAE buffer containing 5 ul of ethidium bromide (0.5ug/ml), it was observed through UV trans-illumination and a picture was taken using a Polaroid Camera.

### c) Heteroduplex Formation

Heteroduplex mobility analysis was carried out according to the HIV-1 Envelope Gene Subtyping Kit for Heteroduplex Mobility

Analysis, Protocol Version 3 (NIH AIDS Research and Reference Program, U.S.A.). The kit consists of 22 reference plasmids from eight HIV-1 subtypes, a detailed protocol and the four sets of PCR primers. Accordingly, to assign the subtype of each PCR-amplified sample, intra-sample and inter-sample heteroduplexes were formed in 500 ul Eppendorf tubes. Intra-sample heteroduplexes provide a baseline heteroduplex pattern with which to compare deliberately formed inter-sample heteroduplexes, where the PCR fragment derived from the unknown strain is reannealed with the corresponding fragment from multiple representatives of each of the previously identified subtypes (Delwart et al., 1995c). To form heteroduplexes then, 5 ul (containing 100-250 ng) of second round PCR product from each sample was mixed with 5 ul of water (for intra-sample heteroduplex formation) or 5 ul of PCR product reference strain (A1-E1) (for inter-sample heteroduplex formation) and 1.1 ul of 10x heteroduplex annealing buffer (1M NaCl, 100 mM Tris-HCl PH 7.8 and 20 mM EDTA) was added. The mixture was heated to 95°C for 2 minutes in PCR machine and cooled rapidly on wet ice. These mixtures were then mixed with 3ul of Ficoll/loading dye (25% Ficoll and 1% Orange G) and loaded onto a 5% non-denaturing polyacrylamide gel (29:1 acrylamide:bisacrylamide; 0.1% ammonium persulfate and TEMED (N,N,N',N'-Tetramethylethylenediamine). The intra-sample heteroduplexes were run on the same gel as that of the inter-sample heteroduplexes so that a visual comparison of heteroduplex mobilities in different lanes could be made. The gels were electrophoresed at 250V for 2.5 hours to compare the 0.5 Kb and

0.7 kb products or at 200V for 4 hours for the 1.2 Kb product in 1x TBE buffer (88mM Tris, 89mM Borate and 2mM EDTA), using a vertical gel electrophoresis apparatus (Model V15.17 GIBCO/BRL, Life Technologies). The gels were stained with 5ul ethidium bromide (0.5ug/ml) for 15 minutes, and the electrophoretic mobility of the heteroduplexes were observed through UV trans-illumination and a picture was taken with Polaroid Camera.

To assign an uncharacterized strain to a known subtype then, the heteroduplex exhibiting the fastest mobilities between the unknown and the most related subtype is taken to indicate the likely subtype of that strain. This is because the mobility of heteroduplexes formed between sufficiently divergent molecules (usually >1 to 2 % mismatches or single-base gaps due to insertion-deletion) is reduced relative to that of the fully complementary homoduplex molecules (Delwart et al., 1995c).

#### 2.3.5 DNA Sequencing of the C2-V3 region.

Plasma samples (4 subtype C; 4 subtype A; 1 subtype D and 4 untypable samples by HMA) were sent to Human Retrovirus Laboratory, Department of Human Retrovirology, University of Amsterdam (Amsterdam, The Netherlands) for DNA sequencing. HIV-1 RNA was isolated from the plasma according to a standard procedure (Boom et al., 1990). Viral RNA was converted to cDNA and then subjected to a nested polymerase chain reaction (PCR) amplifying a 284-bp fragment covering the V3 region of the gp120

gene. First and second PCR conditions were as follows: 35 and 25 cycles of, respectively, 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C, followed by a final incubation for 10 min at 72°C. The amplified products were sequenced with the use of dye-labelled primers (SP6 and T7, Dynamic Direct Cycle Sequencing Kit, Amerhan Life Science) and analyzed with automatic sequencer, ABI (Foster City, CA) 373 A system. Phylogenetic analysis was carried out by the neighbor-joining method of the MEGA program (Saitou and Nei, 1987).

### 3. RESULTS

#### *3.1 HIV Serology and Study Subjects*

In this study, among 150 CSWs from Kazanchis and Tekle-Haimanot health centers, 44.7% (67) were HIV-SPOT positive, while 45.3% (68) were ELISA (Vironostika) positive. The one sample which gave a borderline positive result with the ELISA test was reassessed with the Western Blot and was found to be HIV negative.

The age of all the HIV-1 sero-positive individuals ranged from 17 to 40 years and they had worked as CSWs ranging from 3 months up to 18 years.

#### *3.2 Peptide ELISA for HIV Subtyping*

Peptide sero-typing of the 67 HIV positive samples unambiguously showed 46.3% (31/67) as reactive with subtype C specific peptides. Within these samples, 6.5% (2/31) were reactive with the consensus subtype C peptide only, 16.1% (5/31) with the Ethiopian subtype C peptide only and 77.4% (24/31) with both C peptides used. A relatively large percentage of plasma samples (30.0%; 20/67) showed cross-reactivity between subtype A and C peptides. Additional reactivities were: 3.0% (2/67) with peptides representing subtypes A, C and D, 1.5% (1/67) with subtypes B and D and 1.5% (1/67) with subtypes C and D (OD > 0.9) (Table. 1). 9.0% (6/67) of the samples were found to be

indeterminate (OD= 0.700-0.900) while 9.0% (6/67) were non-reactive (OD < 0.7). No sample reacted exclusively with peptides representing subtypes A, B or D.

### 3.3. HMA Analysis for HIV Subtyping

By using four different combinations of first and second round PCR primers, amplification was made possible for all 67 HIV-1 samples (Table 2). Initially, ED3/ED14 and ED31/ED33 were used as first and second round PCR primers, respectively. These amplified the majority 43/67 of the samples. HMA performed with these primer sets identified 81.4% (35/43) samples as subtype C (Fig.6) and 2.3% (1/43) as subtype D (Fig.7). The rest 16.3% (7/43) were non-typable. The second primer set, ED5/ED12 and ED31/ED33, were used to amplify those samples which were not previously amplified. Of these 24 samples, 33.3% (8/24) were identified as subtype C, while the rest 66.7% (16/24) were non-typable by HMA. The third primer combination, ED3/ED14 and ED5/ED12, amplified 15/23 samples and all the amplified samples were identified as subtype C. The fourth primer combination, ED5/ED12 and ES7/ES8 amplified all the 8 unamplified samples, and of these 4 were identified as subtype A (Fig. 8). A total of 4 samples (about 6%) remained untypable by HMA. In general, HMA identified 86.5% (58/67) as subtype C, 6% (4/67) as subtype A and 1.5% (1/67) as subtype D. 6% (4/67) of the PCR products were non-typable by this method.

Table 1. Peptide ELISA results. (Abbreviations:

I= Indeterminate, NR= Non-Reactive).

SUBTYPE BY PEPTIDE ELISA	TOTAL (N/67)
A	0
B	0
C	31
D	0
A/C	20
A/C/D	2
C/D	1
B/D	1
I	6
NR	6

Table 2. Results of Heteroduplex Mobility Assay.

(Abbreviations: NT= Nontypable)

PCR PRIMER COMBINATION	PCR AMPLIFICATION  (positive)	SUBTYPES				
		A	B	C	D	NT
ED3/ED14, ED31/ED33 (0.5-kb)	43/67	0	0	35/43	1/43	7/43
ED5/ED12, ED31/ED33 (0.5-kb)	24/24	0	0	8/24	0	16/24
ED3/ED14, ED5/ED12 (1.2-kb)	15/23	0	0	15/15	0	0
ED5/ED12, ES7/ES8 (0.7-kb)	8/8	4/8	0	0	0	4/8
TOTAL SUBTYPES BY HMA		4/67 (6%)	0/67 (0%)	58/67 (86.5%)	1/67 (1.5%)	4/67 (6%)



↓

E1	D3	D1	C2	C1	B1	A2	A1	X	M	E1	D3	D1	C2	C1	B1	A3	A2	A1	z
+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
X	X	X	X	X	X	X	X	X	X	z	z	z	z	z	z	z	z	z	z

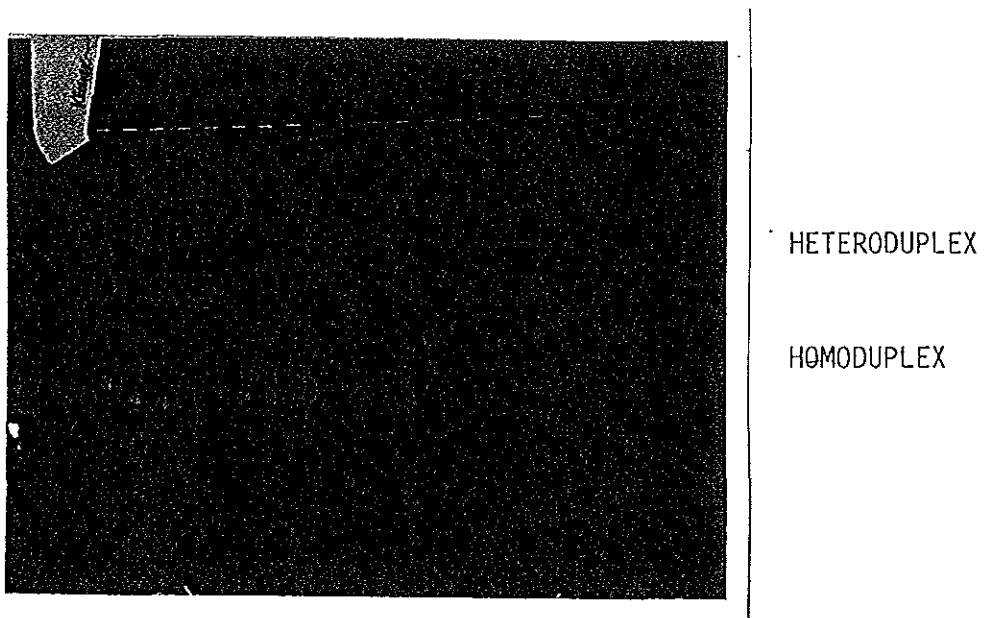


Figure 7. HMA of sample KS3.71 showing subtype D heteroduplex mobility. 0.5-kb C2-C3 region of the *env* gene of HIV-1 strains and different reference subtypes (A1, A2, B1, C1, C2, D1, D3 and E1) were analyzed on a nondenaturing 5% polyacrylamide gel in TBE buffer. KEY (X= KS3.71 (KS=Kazanchis), M= Marker)

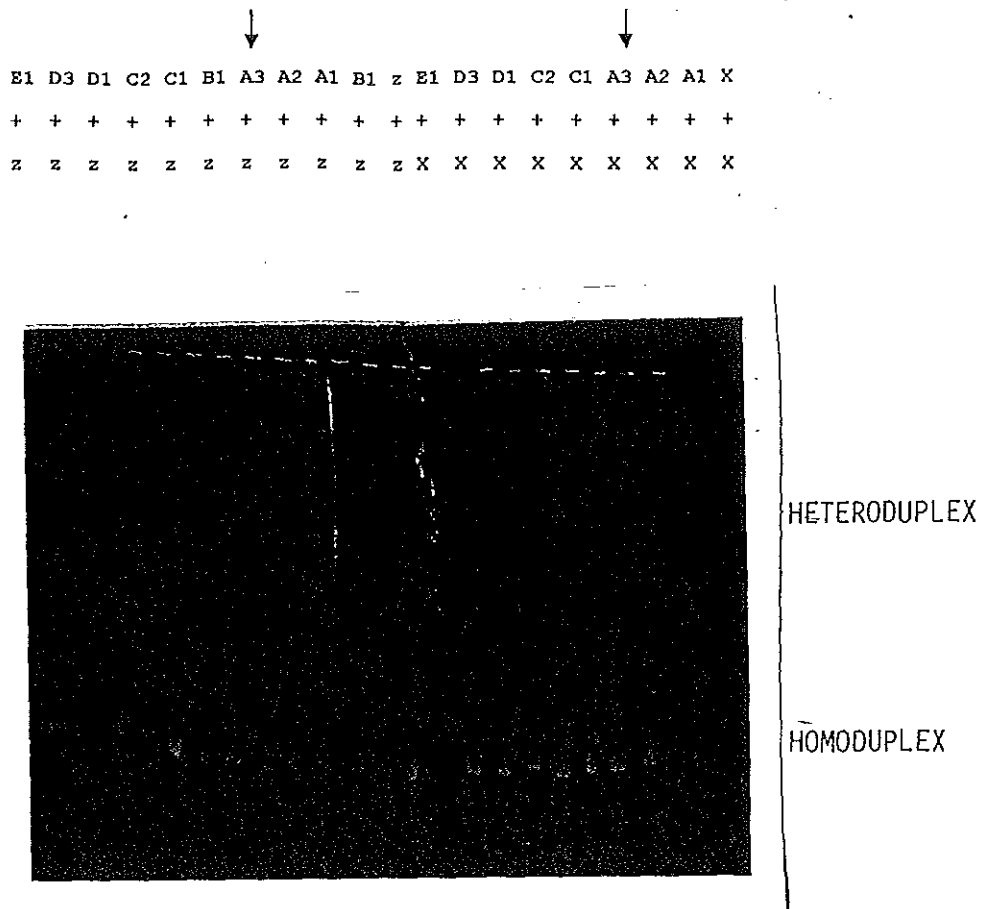


Figure 8. HMA of sample KS3.13 and KS3.20 showing subtype A heteroduplex mobility. 0.7-kb V3-V5 region of the *env* gene of HIV-1 strains and different subtypes (A1, A2, B1, C1, C2, D1, D3 and E1) were analyzed on a nondenaturing 5% polyacrylamide gel in TBE buffer. KEY (Z= KS3.13 and KS3.20 (KS= Kazanchis)).

When peptide ELISA results of the 67 samples were compared with HMA, there was only 43.3% (29/67) agreement (Table 2). HMA characterized the majority of the A/C cross-reactive samples as subtype C (75%), a sizeable fraction as subtype A (20%) and the rest as non-typable (5%). All the A/C/D cross-reactive, C/D cross-reactive, non-reactive (except for one sample) and indeterminate samples by peptide ELISA were typed subtype C by HMA. The B/D cross-reactive sample was subtype D by HMA.

### *3.4 DNA Sequencing*

Sequencing of the V3 region was performed on a total of 13 samples for quality control of HMA. The following samples were sequenced: 4 subtype C, 4 subtype A, 1 subtype D, 3 untypable and 1 aberrant samples by HMA. Of these, 2/4 subtype C, 3/4 subtype A, 2/3 untypable and 1 aberrant samples by HMA were all found to cluster in subtype C (Fig. 9). One subtype D sample by HMA was found to belong to the same subtype. Four samples were untypable. From the amino acid sequence alignment (Fig. 10), all V3 loops were 35 amino acids long and showed the characteristic C-subtype pattern of GPGQT motif at the apex. The single D-subtype sample showed the consensus D subtype apex (GPGQAL).

Phylogenetic analysis of the sequenced samples based on the complete deletion method of the MEGA program clustered them all in the Main group of Ethiopian subtype C sequences (Data not shown).

Table 3. Comparison of peptide ELISA results with HMA.

(Abbreviations: I= Indeterminate, NT = Nontypable,  
NR = Non-Reactive).

SUBTYPE BY PEPTIDE ELISA	SUBTYPE BY HMA					
	A	B	C	D	NT	TOTAL
A	0	0	0	0	0	0
B	0	0	0	0	0	0
C	0	0	29	0	2	31
D	0	0	0	0	0	0
A/C	4	0	15	0	1	20
A/C/D	0	0	2	0	0	2
C/D	0	0	1	0	0	1
B/D	0	0	0	1	0	1
I	0	0	6	0	0	6
NR	0	0	5	0	1	6
TOTAL	4	0	58	1	4	67



LOOP APEX

Consensus:	IVIRSENLTRHAKIIIVQLKEPVEIVCTRPNNTRKSMRIGPGQTFYATGDIIGDIRQAHCNISEETWNKTLQEVGKKLQEHFENKTI	D
KS3.71	-I-----ISD-----N-S-T-N---Y---Q-IH-----AL-T-KI--GDI-----RAE-----QVA---GAL-NKTEL	C
TS3.09	VI-----QFN-S--H-----I-----G-----Y-NVNGSK--A--Q-AN--KG--N-TPI	
KS3.20	-I-----KHIT-T-----E--N--W-Y---GKA--D-----R--E-----R--	
KS3.32	MI--C-----D-----N-E-----RY---R-----L---GL--G---D--D-K--E---DVGRELVTLPH-RSI	
KS3.41	--V-V--D-T-T-----Q-N--G---E-I-----Y-----K-----K-KE--G---HK-IS	
KS3.13	-----D-V-T--ML-D-----IV-----E-----QK-----R--N-----	
TS3.10	-I-----FN-S-Q-N---D---GIG-----A-----K-K-----G-KE--GKL-----	
TS3.66	-----D--T--H--K--GI---G---R-----K-NGSE--E--G-SE--RQ---G-A-	
KS3.26	-----H-TK--I-----A-----K--D--K---E-----S-	

Figure 10. Predicted amino acid sequences of the V3 region of gp120 V3 regions of 9 plasma sequences on 1997 Addis Ababa CSW, as compared to an Ethiopian consensus sequence. The subtype D sequence is shown first (KS3.71), followed by the subtype C sequences. Dashes (-) indicate amino acid identity with consensus sequence.

## 4. DISCUSSION

The present study demonstrates that HIV-1 subtype C continues to predominate in Addis Ababa, Ethiopia. The presence of subtype C is also reported in the neighboring countries of Ethiopia: Kenya, Somalia and Djibouti (Louwagie et al., 1995). However, in these countries HIV-1 subtype C represents only a fraction of the prevalent subtypes (Janssens et al., 1997). The situation in Addis Ababa parallels the situation in countries with relatively recent HIV-1 epidemics, like India, Brazil and South Africa, where again HIV-1 subtype C predominates (Louwagie et al., 1993). The cumulative data presently available in Ethiopia, where the estimated number of HIV-1 infected individuals is around 2 million (Ethiopian Ministry of Health, 1996), indicate that the HIV-1 epidemic is most probably to a large extent of the C subtype (Ayehunie et al., 1990; Ayehunie et al., 1991; Ayehunie et al., 1993; Sherefa et al., 1994a, b and 1997; Johansson et al., 1995; Salminen et al., 1996; Abebe et al., 1997). According to preliminary calculations of WHO, 36-50% of all HIV infected individuals in the world are infected with subtype C, making subtype C the most prevalent in the world (Esparza, personal communication). Altogether, the above data underline the general importance of vaccine development against HIV-1 subtype C.

This study also presents the first evidence of an HIV-1 D

subtype in Addis Ababa. This subtype was identified from a 25 year old CSW with 3 years of commercial sex work experience attending the Kazanchis Health Center. A previous study has shown the presence of a single HIV-1 subtype A out of 94 sequenced sera (C subtype) collected from various risk groups from Addis Ababa between 1988 and 1995 (Abebe et al., 1997). These findings indicate the presence, though at substantially low frequencies (1.3%), 2/158 sequenced samples, ENARP) of HIV-1 subtypes A and D in Addis Ababa. In contrast, studies neighboring Kenya and Djibouti (Louwagie et al., 1995) and other East African countries, like Uganda (Bruce et al., 1994) and Tanzania (Robbins et al., 1996) have found subtypes A and D to be the most common HIV-1 subtypes.

The reason for the predominance of subtype C among CSW in Addis Ababa (98.4%) can not be given with absolute certainty. However, it can be speculated that this subtype has managed to quickly saturate the CSW network in the mid 1980's. Already in 1988 prevalences were found in the order of 30-38% in CSW from towns along the main road from the Assab harbor to Addis Ababa (Mehret et al., 1990a). In 1989 the HIV-1 prevalence in red light house CSW from Addis Ababa (Mehret et al., 1990b) were identical to the ones detected in the present study (1989 red light house CSW: 43.8% versus 1997 CSW: 44.7%). The fact that the first HIV-1 positive sample in Addis Ababa was detected in 1984 (Tsega et al., 1988) and saturation of the CSW network was established only 5 years later supports the hypothesis of a fast spread of the

epidemic. The route of HIV-1 infection in Ethiopia is in far majority by heterosexual contacts, alternative networks being minor contributors to the spread of the epidemic. This reduces the chance of introduction of other subtypes creating different subtype epidemics in different networks, as reported in South Africa (van Hermelen et al., 1997) or Thailand (Ou et al., 1993).

In this study, the WHO algorithm for HIV-1 subtyping which includes initial screening by subtype specific V3 peptide ELISA followed by the validation of the serological results by HMA and finally DNA sequencing of nontypable or ambiguous results obtained by HMA (Wasi et al., 1995) was performed for the first time in Ethiopia.

Accordingly, with peptide ELISA, the use of a tailor-made "Ethiopian consensus" C peptide has partly increased the peptide ELISA sensitivity: 53/66 (80.3%) C subtypes were identified, as compared to 47/66 (71.2%) by the WHO EVA programme consensus peptide. However, in terms of specificity, the peptide ELISA results showed a high rate of serum cross-reactivity 24/67 (36%) with the panel of V3 peptides tested. This appears to be inevitable, since gp120 V3 sequence homologies between different subtypes have been demonstrated (Cheinsong-Popov et al., 1994). Especially, this study confirms the wide cross-reactivity between gp120 V3 subtype A and C peptides used for peptide ELISA, as reported earlier (Zwart et al., 1993). The increase of OD cut off value from 0.700 to 0.900, based on higher antibody titers in

Africans, did not significantly influence the interpretation of our peptide ELISA results.

With HMA, a relatively more reliable and definitive determination of HIV-1 subtypes was achieved. This method unambiguously identified 58/67 plasma samples as subtype C and 1/67 as subtype D. Four samples were wrongly identified as subtype A and another four could not be subtyped unambiguously, although PCR products were generated of the proper size. The incorrect A subtyping results are attributed to the use of the PCR primers ES7/ES8, which indeed were reported to cause some subtyping errors in discriminating HIV-1 subtype A from C (Delwart, personal communication).

Based on the results obtained in this study, the following algorithm for HMA aided HIV-1 subtyping in Ethiopia can be proposed: 1) ED3/ED14 followed by ED5/ED12; 2) ED3/ED14 followed by ED31/ED33 or 3) ED5/ED12 followed by ED31/ED33. This order of primer combination allowed better amplification and subtyping of HIV-1 by HMA. The use of ES7/ES8 primer is not recommended.

Phylogenetic analysis revealed that the 1997 Addis Ababa CSW gp120 V3 sequences belong to the diversified Main Group of Ethiopian subtype C sequences, as defined by Abebe et al., 1997 suggesting that this highly divergent variants of HIV-1 subtype C are still the dominant forms in the population.

In general for large-scale subtyping in Addis Ababa, it is important to consider the following practical points. Though peptide ELISA uses the simple, rapid and widely available enzyme-linked immunosorbent assay technology, it requires the generation of synthetic peptides and is affected by cross-reactivity especially in areas where A and C subtypes are co-circulating. In a previous study (Abebe et al., 1997) the only HIV-1 subtype A sample identified (as confirmed by V3 sequencing), was incorrectly diagnosed as subtype C by peptide ELISA (Abebe, personal communication). Therefore, a first screening by peptide ELISA may not be appropriate in the Ethiopian setting. On the other hand, HMA allowed a more definitive determination of HIV-1 subtypes. Overall, HMA is more appropriate and is recommended for subtype identification in Ethiopia. Whereas, DNA sequencing although it remains the gold standard for HIV-1 subtyping, this technology is not always practical in a developing country setting.

In conclusion, in an attempt to assess the prevalence of non-C subtypes among CSWs in Addis Ababa, this study, further confirmed the presence of subtype C as the major subtype in the samples analyzed. The presence of subtype D, though at substantially lower frequency, is also indicated. Therefore, it is obvious that the Addis Ababa CSWs has already been saturated with the C-subtype and it would become difficult for other HIV-1 subtypes to enter into the Addis Ababa CSWs.

Since this study was carried out in Addis Ababa CSW only, further work is required to assess the existence and distribution of other subtypes in other parts of the country, especially in the border regions. For this purpose, a more intensive subtyping within a small number of subjects by HMA and final DNA sequencing of some samples selected after screening with HMA will facilitate a rapid and efficient way of identifying the HIV-1 diversity present within large population. Furthermore, assessing the env V3 regions of CSW who are probably infected by HIV-1 only recently in this study, and comparing to V3 regions of historical isolates from Addis Ababa might give an insight to the molecular evolution of HIV-1 subtype C in Ethiopia. In addition since there is a report only recently about an Ethiopian A/C recombinant (Sherefa et al., 1998) and we have based all our subtyping results up to now on gp120 V3 only (peptide ELISA, HMA, and sequencing) it might be that we misinterpret the contribution of A or A/C recombinants to the epidemic in this country. Therefore, gag sequencing of certain random samples from this or another study is suggested. Finally, data are still missing on two samples (KS3.12 and TS3.53) and we need to subtype them, either by V3 or gag sequencing. Knowledge about the genetic diversity and geographical distribution of HIV-1 subtypes in Ethiopia can contribute to the efficacy of future vaccine implementations and diagnostic assays.

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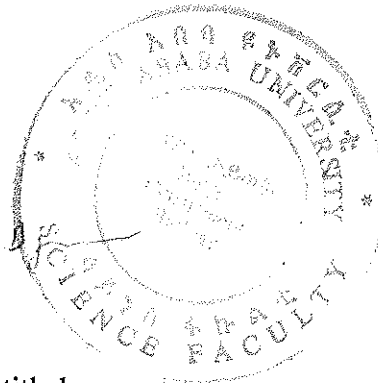
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This is to certify that this copy of the M.Sc thesis entitled:

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