HIV-1 subtype C in Ethiopia: genotypic and phenotypic variation
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CHAPTER III

HIV-1 subtype C in commercial sex workers in Addis Ababa, Ethiopia

Mintewab Hussein, Almaz Abebe, Georgios Pollakis, Margreet Brouwer, Beyene Petros, Arnaud L. Fontanet, and Tobias F. Rinke de Wit

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HIV-1 Subtype C in Commercial Sex Workers in Addis Ababa, Ethiopia

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Summary: In this study, we have investigated the diversity of the current HIV-1 strains circulating in Addis Ababa, Ethiopia; in addition, we have evaluated the applicability of peptide enzyme-linked immunosorbent assay (ELISA) and heteroduplex mobility assay (HMA) for HIV-1 subtyping. Previous studies have indicated that HIV-1 subtype C is the major subtype present in HIV-positive samples collected from various risk groups between 1988 and 1995 in Addis Ababa. To assess the possible influx of new HIV-1 subtypes, 150 commercial sex workers (CSW) reporting in 1997 to two Health Centers in Addis Ababa were enrolled in an unlinked anonymous cross-sectional study. Subtyping was performed according to the World Health Organization algorithm of peptide ELISA, followed by HMA and DNA sequencing. As a result, the HIV-1 prevalence among these CSWs was found to be 45% (67 of 150). Of the 67 samples, 66 contained HIV-1 of subtype C and only one was of subtype D. This confirms the persistent overall presence of HIV-1 subtype C in Addis Ababa and a low influx of other subtypes into this location. Key Words: HIV-1 subtype—Ethiopia—Commercial sex workers.

The HIV-1 genome is characterized by extensive variation, especially in the gp120 env gene. DNA sequencing and phylogenetic analyses have led to the identification of at least 10 different subtypes (A–J) within the major group of HIV-1 viruses, group M (1–3). Furthermore, highly divergent viruses can be identified within the outlier group of HIV-1 viruses, group O (4). Between subtypes, the nucleotide sequence divergence of env is up to 30% (1). The spread of the different HIV subtypes around the world is a dynamic process. In Africa, existence of all genetic subtypes has been confirmed, with a preference for subtypes A, C, and D (1,5). The presence of this multitude of subtypes indicates the extensive divergence that HIV-1 has accumulated in the African continent (6–9). This implies a serious challenge for future vaccine-based prevention efforts.

In Ethiopia, since the first report of HIV-1–positive sera in 1984 (10) and the first AIDS cases in 1986, the epidemic has spread to reach a prevalence in 1997 of between 14% and 20% in urban pregnant women, 47% and 59% in commercial sex workers (CSW; Ethio-Netherlands AIDS Research Project [ENARP] sentinel survey 1997) and 7% in blood donors (Ethiopian Red

The Ethiopian HIV-1 epidemic has been shown to be dominated by subtype C (11-19). 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 (11,12). Other studies done by using peptide ELISA (13-15) and DNA sequencing (16) further confirmed the presence of subtype C as the major HIV subtype in Addis Ababa. Recently, the sequencing of 94 viral isolates collected between 1989 and 1995 from various risk groups in Addis Ababa showed again only HIV-1 C subtypes, with the exception of one subtype A HIV-1 isolate from a pregnant woman in 1995 (17). Furthermore, within these cited 93 HIV-1 subtype C isolates, two different subclusters were detected, suggesting the possibility of two independent introductions of HIV-1 C subtype viruses into Ethiopia (17). 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 (16,18). Finally, another full-length Ethiopian 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 man in October 1991 (19).

The present study assesses the molecular characteristics of the env genes of 67 HIV-1-positive plasma samples collected in 1997 from CSWs in Addis Ababa. The rationale for this study was twofold. First, it was intended to gain an insight in the possible influx of new, non-C HIV-1 subtypes in Addis Ababa. CSWs were selected, because they are likely to be the first targets of possible introduction of new subtypes into the population of Addis Ababa. Second, the applicability of the WHO algorithm for HIV-1 subtyping, namely, gp120 V3 peptide ELISA, followed by HMA and finally DNA sequencing, was tested in an Ethiopian context.

METHODS

Study Population

Blood samples treated with ethylenediaminetetraacetic acid of 150 CSWs seen for symptoms of sexually transmitted diseases at Tekle-Haimanot (n = 75) and Kazanchis (n = 75) Health Centers in Addis Ababa were collected. Age and number of years spent as a CSW were recorded at the time of blood collection. Study subjects were enrolled in order of arrival (convenient sampling) and HIV-1 tests were performed in an unlinked anonymous way, according to WHO guidelines (20). Samples collected were coded 97K* (for Kazanchis, 1997) or 97T* (for Tekle-Haimanot, 1997). Syphilis serology was performed by Treponema pallidum particle agglutination assay (TPPA, Sero­dia-TPPA, Fujirebio, Japan) and rapid plasma reagin assay (RPR, BioMerieux, France), according to the manufacturers' instructions. Syphilis results were returned to the Health Centers and benzathin/ penicillin treatment was provided to the study participants for free.

HIV Screening and Confirmatory Assays

HIV screening was performed by HIV-SPOT (Genelabs Diagnostics, Singapore) and ELISA (Vironostika HIV Uni-Form II, Organon Teknika, The Netherlands). Discrepant results were confirmed by a Western Blot Assay (HIV BLOT 2.2, Genelabs Diagnostics, Singapore).

Peripheral Blood Mononuclear Cell Isolation and DNA Extraction

Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll-Hypaque density gradient. After two washes in Earle’s heparin new born calf serum (EHN) medium, cells were pelleted and stored at -80°C. DNA was extracted as previously reported (21).

Peptide Enzyme-Linked Immunosorbent Assay

Four peptides derived from the V3 loop region of HIV-1, from the WHO EVA program were used as antigens (Fig. 1). A fifth peptide was a tailor-made Ethiopian subtype C peptide, based on previous sequencing data (17). The amino acid sequences of the peptides were as follows: subtype A, KSVHGPGQAFYAT; subtype B, KSHHGPG­RAYFTT; subtype C, KSRIGPGQTFTYAT; subtype C (Ethiopia), KSIRGPGQTFTYAT; and subtype D, QRTHPGQALYTT. Ninety-six-well microplates (Nunc-Immuno Plate, Maxisorp Surface, Life Technologies, Roskilde, Denmark) were coated with 100 ng of peptide per well in 100 μl phosphate-buffered saline (PBS) overnight at room temperature (RT). Nonspecific binding sites were blocked with 150 μl of blocking buffer (100 μl/well) and incubated for 1 hour at 37°C. Plasma samples of 100 μl, diluted 1:100 with blocking buffer were dispensed in duplicate and incubated for 1 hour at 37°C. Bound antibodies were detected by goat anti-human horseradish-peroxidase–labeled conjugate (Kirkegaard and Perry Laboratory, Inc.), diluted 1:10,000 in PBS, 7% skimmed milk (100 μl/well) and incubated for 1 hour at 37°C. Substrate was added: orthophenylenediamine dihydrochloride (Abbott Laboratories, Abbott Park, IL, U.S.A.), 100 μl/well and incubated for 10 minutes. The reaction was stopped with 100-μl 1 N H₂SO₄ and the optical density (OD) was measured at 450nm.

The cutoff OD value of the stated peptide ELISA is 0.700 on samples from HIV-1–positive white persons, but because of high plasma levels of nonspecific antibodies in Ethiopians (22), the cutoff values were increased to OD = 0.900, based on control experiments. Those reactions with OD readings between 0.700 and 0.900 were considered as indeterminate and those below 0.7 were considered nonreactive.

Polymerase Chain Reaction and Heteroduplex Mobility Assay

Genomic DNA (5 μl, equivalent to 100,000 cells) was first amplified using the primers ED3/14 and/or ED5/12 (22), under the following cycling conditions: 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). The second round amplification was performed with 2 μl of the first round product, using primers ED5/12, ED3/33, and/or ES7/8 (23) and the same cycling conditions except...
FIG. 1. Alignment of predicted HIV-1 gp120 V3 amino acid sequences of 17 bulk plasma samples drawn in 1997 from commercial sex workers (CSW) in Addis Ababa (GenBank accession numbers: AF160813–AF160829), compared with 13 bulk plasma samples from 1989/1990 CSW (labeled with year of sample collection (89' or 90'), followed by sample identification), as published (17). CONSC, Los Alamos 1998 subtype C consensus sequence; dot (.), identical residue as consensus sequence; dash (−), gap; question mark (?), ambiguous residue. The apex of the V3 loops are in **bold**.

that the five cycles were reduced to three and the 30 cycles increased to 32 cycles. Reaction conditions were 10 pmol of each primer, 0.2-mM deoxynucleoside (dNTP), 3-mM MgCl₂, 1 mM MgCl₂-free polymerase chain reaction (PCR) buffer (Promega, U.S.A.) and 2.5 U Taq-DNA polymerase (Promega, Madison, WI, U.S.A.) in a final volume of 50 μl. The algorithm for nested PCR was as follows:

1. ED3/14 followed by ED31/33
2. (On all negative results of PCRs of step 1): ED5/12 followed by ED31/33
3. (On all ambiguous results of steps 1 and 2): ED3/14 followed by ED5/12

Heteroduplex molecules were obtained by mixing the PCR-amplified DNA fragments of subject studies with PCR amplificates of reference strains. Denaturation was at 95°C for 2 minutes and renaturation by rapid cooling on ice. The reference strains used in this study were A1 (RR20, Rwanda), A2 (IC144, Ivory Coast), A3 (SF170, Rwanda), B1 (BR20, Brazil), C1 (MA559, Malawi), C2 (ZM18, Zambia), C3 (IN868, India), C4 (BR25, Brazil), D1 (UG21, Uganda), D3 (UG46, Uganda) and E1 (TH112, Thailand). The reaction was performed in 1 M NaCl, 100 mM Tris-Cl (pH = 7.8), and 20 mM ethylenediaminetetraacetic acid (EDTA) in a final volume of 1.1 μl. The heteroduplex formation was assessed by nondenaturing polyacrylamide gel electrophoresis at 250 V for 2.5 hours for ED31/33 or ES7/8 PCR products or at 200 V for 4 hours for ED5/12 products. Gel composition: 5% polyacrylamide in 1 × TBE buffer (88 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA), staining with ethidium bromide (25). GenBank accession numbers of the obtained sequences are AF160813–AF160829. GenBank accession number of the 1989/1990 CSW sequences used for comparisons are as follows: sequence 89*1653 = U87827, sequence 89*1361 = U87874, sequence 89*229 = U87828, sequence 89*2781 = U87829, sequence 89*2841 = U87881, sequence 89*2733 = U87872, sequence 89*1896 = U87883.
sequence 90*589 = U88732, sequence 90*1290 = U88779, sequence 90*1001 = U88730, sequence 90*1296 = U88731, sequence 90*601 = U88778, and sequence 90*1252 = U88780.

RESULTS

Among 150 CSWs who were seen at Addis Ababa Kazanchis and Tekle-Haimanot Health Centers, 67 (45%) were HIV-1-positive. The age of these HIV-1-positive CSWs ranged from 17 to 40 years (mean, 25 years). Some 37% (25 of 67) of them had a history of syphilis infection, as determined by a positive TPPA result and 12% (8 of 67) reported with a recent syphilis infection, as determined by a positive RPR test result.

As presented in Table 1, HIV-1 subtyping by peptide ELISA of the 67 HIV-positive plasma samples unambiguously showed 46% (31 of 67) exclusively reacting with either one or both subtype C peptides. Within these samples, 3% (1 of 31) were reactive with the WHO consensus subtype C peptide only, 16% (5 of 31) with the Ethiopian subtype C peptide only and 81% (25 of 31) with both C peptides used. A relatively large percentage of plasma samples (30%; 20 of 67) showed cross-reactivity between subtype A and subtype C peptides.

Table 2 shows a comparison of peptide EUSA and HMA test results on 67 HIV-1-positive plasma samples from commercial sex workers. Among 150 CSWs who were seen at Addis Ababa Kazanchis and Tekle-Haimanot Health Centers, 67 (45%) were HIV-1-positive. The age of these HIV-1-positive CSWs ranged from 17 to 40 years (mean, 25 years). Some 37% (25 of 67) of them had a history of syphilis infection, as determined by a positive TPPA result and 12% (8 of 67) reported with a recent syphilis infection, as determined by a positive RPR test result.

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Plasma samples with other reactivities were: 2 of 67 samples (30%; 20 of 67) showed cross-reactivity with both C peptides used. A relatively large percentage of plasma samples (30%; 20 of 67) showed cross-reactivity between subtype A and subtype C peptides. Plasma samples with other reactivities were: 2 of 67 (3%) with peptides representing subtypes A, C and D; 1 of 67 (2%) with subtypes B and D and 1 of 67 (2%) with subtypes C and D. Nine percent of the samples (6 of 67) were found to be indeterminate (OD = 0.700–0.900) whereas another 9% (6 of 67) were nonreactive (OD < 0.700). No sample reacted exclusively with peptides representing HIV-1 subtypes A, B or D.

For HMA, different combinations of primers, as described in the Methods section, resulted in the successful amplification of all 67 (99%) HIV-1-positive samples (Table 2). HMA identified 87% (58 of 67) as subtype C, 6% (4 of 67) as subtype A and 2% (1 of 67) as subtype D; 6% of the PCR products (4 of 67) could not be typed by this method.

When peptide ELISA results for the 67 specimens were compared with HMA, there was only 43% (29 of 67) agreement (Table 1). HMA characterized the majority of the peptide ELISA A/C cross-reactive samples as subtype C (15 of 20), a sizable fraction as subtype A (4 of 20) and 1 A/C cross-reactive sample could not be typed (97T*9). All the peptide ELISA A/C/D cross-reactive, C/D cross-reactive, nonreactive and indeterminate samples were determined subtype C by HMA. The B/D cross-reactive sample (Table 1) appeared to be subtype D by HMA.

The plasma samples that could not be subtyped by peptide ELISA or HMA (n = 3), or that gave ambiguous results when comparing these methods (n = 4), were selected for DNA sequencing of the HIV-1 gp120 V3 regions. In addition, 9 plasma samples unambiguously typed HIV-1 C-subtype by HMA and the 1 sample typed HIV-1 D subtype by HMA were sequenced for confirmational purposes. Results are shown in Figure 1, in comparison with gp120 V3 sequences obtained from CSW from the same locale 7 to 8 years earlier (16). This comparison illustrates the accumulation of mutations in the gp120 V3 regions of CSW HIV-1 isolates over time.

Figure 2 shows an unrooted phylogenetic analysis of the gp120 V3 nucleotide sequences, in conjunction with consensus sequences obtained from Los Alamos, USA (1) and gp120 V3 sequences from CSW from the same locales in 1989/90 (n = 13), 1992 (n = 24), and 1995 (n = 19). Of the 17 sequenced plasma samples 16 clustered with HIV-1 consensus C and 1 (97K*71) with consensus D. There was no clustering of samples collected from 1997 CSW with samples from CSW collected at any earlier time point. Rather, the HIV-1 gp120 V3 sequences had a completely mixed topology as regards year of sample collection.

More in detail, the phylogenetic analyses revealed that the 4 samples determined to contain HIV-1 subtype A viruses by HMA (97K*12, 97K*20, 97K*26 and 97T*66) did in fact contain HIV-1 subtype C virus (Fig. 2). Of the 4 samples, which could not be typed by HMA (97K*32, 97T*9, 97T*10, 97T*53), 3 could be determined as HIV-1 subtype C by sequencing (Fig. 1); only 97T*53 plasma could not be PCR amplified with the

Table 1. Comparison of peptide ELISA and HMA test results on 67 HIV-1-positive plasma samples from commercial sex workers

<table>
<thead>
<tr>
<th>Subtype by peptide ELISA</th>
<th>Subtype by HMA</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>0</td>
</tr>
<tr>
<td>A/C</td>
<td>0</td>
</tr>
<tr>
<td>A/CD</td>
<td>0</td>
</tr>
<tr>
<td>C/D</td>
<td>0</td>
</tr>
<tr>
<td>B/D</td>
<td>0</td>
</tr>
<tr>
<td>NR</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
</tr>
</tbody>
</table>

* Numbers of samples reactive with the Ethiopian consensus C peptide only are indicated in parentheses; the numbers of samples reactive with the WHO consensus C peptide only are indicated in square brackets; all other samples reacted positively with both HIV-1 C peptides in the peptide ELISA. ELISA, enzyme-linked immunosorbent assay; HMA, heteroduplex mobility assay; NT, nontypable by HMA; I, indeterminate; NR, nonreactive.
### TABLE 2. HMA results on 67 HIV-1-positive plasma samples from commercial sex workers

<table>
<thead>
<tr>
<th>PCR primer combinations</th>
<th>First-round PCR</th>
<th>Second-round PCR</th>
<th>Amplification frequency</th>
<th>HMA result (subtype)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
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<td>B</td>
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<td></td>
<td>C</td>
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<tr>
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<td></td>
<td></td>
<td>D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NT</td>
</tr>
<tr>
<td>ED3/14</td>
<td>ED31/33</td>
<td></td>
<td>43/67 (64%)</td>
<td></td>
</tr>
<tr>
<td>ED5/12</td>
<td>ED31/33</td>
<td></td>
<td>24/24 (100%)</td>
<td></td>
</tr>
<tr>
<td>ED3/14</td>
<td>ED5/12</td>
<td></td>
<td>15/23 (65%)</td>
<td></td>
</tr>
<tr>
<td>ED5/12</td>
<td>E57/8</td>
<td></td>
<td>8/8 (100%)</td>
<td></td>
</tr>
<tr>
<td>Total subtypes by HMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>4/67 (6%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0/67 (0%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>58/67 (87%)</td>
<td>1/67 (2%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4/67 (6%)</td>
</tr>
</tbody>
</table>

*Polymerase chain reaction (PCR) results are shown in order of the nested PCR algorithm used for this study, as described in the Methods section. HMA, heteroduplex mobility assay; NT, nontypable by HMA.

Primers used for sequencing. However, this sample gave unambiguous peptide ELISA results, with strong reactivities (OD > 2.300) on C subtype peptides and complete absence of reactivity on any other peptide tested. The plasma sample determined as HIV-1 subtype D by HMA (B/D by ELISA) was confirmed to be of subtype D by DNA sequencing (97K*71). Finally, for quality control of HMA, 9 random samples were sequenced (97K*08, 97K*13, 97K*41, 97K*63, 97T*08, 97T*14, 97T*33, 97T*56 and 97T*72) and all were confirmed to contain HIV-1 subtype C virus (Fig. 1). In conclusion, among the 67 HIV-1-positive plasma samples of this study, 66 appeared of subtype C and only 1 of subtype D. Thus, using DNA sequencing results as a gold standard
and HMA results when sequencing results were not available, the sensitivity of the peptide ELISA for identifying HIV-1 subtype C strains was 54 of 66 (82%).

Table 3 puts the overall diversity of the gp120 V3 regions of the 17 Addis Ababa CSW from 1997 in the context of diversity calculated from previously sequenced HIV-1 from CSW from the same locale. Data were included from a previous study performed by our group (17). It can be concluded that there is a significant increase over time of the overall means of both the proportion of synonymous substitutions per potential synonymous site (\(dS\)) and the proportion of nonsynonymous substitutions per potential nonsynonymous site (\(dN\)). Thus, the HIV-1 C subtype epidemic in Addis Ababa is still accumulating diversity in 1997.

**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) (26). However, in these countries HIV-1 subtype C represents only a fraction of the prevalent subtypes (27). The situation in Addis Ababa parallels the situation in countries with relatively recent HIV-1 epidemics, like India and South Africa, where again HIV-1 subtype C predominates (28). The cumulative data presently available on Ethiopia, where the estimated number of HIV-1–infected individuals is approximately 2.6 million (Ethiopian Ministry of Health, 1997), indicate that the HIV-1 epidemic is most probably to a large extent of the C subtype (11–19). According to UNAIDS, 48% of HIV-1–positive individuals in the world (equivalent to an estimated 14,680,000 subjects) were infected with HIV-1 subtype C by the end of 1997, making subtype C the most prevalent worldwide (Q. Abdool Karim, Geneva AIDS Conference, 1998). Altogether, the above data underline the general importance of vaccine development targeting specifically HIV-1 subtype C.

This study presents the first evidence of a HIV-1 D subtype in Addis Ababa. This subtype was identified from a 25 year old CSW attending the Tekle-Haimanot Health Center. In a previous study we demonstrated the presence of HIV-1 subtype A virus in a 23 year old pregnant woman reporting at the Tekle-Haimanot Health Center in 1995; all other 93 sera from various risk groups from Addis Ababa contained HIV-1 subtype C viruses (17). In summary, these findings indicate the presence, though at substantially low frequencies, of HIV-1 subtypes A and D in Addis Ababa. In contrast, studies in neighboring Kenya, Djibouti and other East African countries, like Uganda (29) and Tanzania (30) have found subtypes A and D to be the most prevalent HIV-1 subtypes (26,30–32).

The reason for the predominance of subtype C in Addis Ababa cannot be given with absolute certainty. However, it can be speculated that this subtype, when introduced into the country in the early and mid-1980s, has first rapidly saturated the CSW network. In support of this, already in 1988, only 4 years after the first HIV-1–positive sera were detected in Ethiopia (10), a HIV-1 prevalence of 30% to 38% was found in CSW from towns along the main road from the Assab harbor on the Red Sea, to Addis Ababa (33). And in 1989 the HIV-1 prevalence in CSW from Addis Ababa (44%) was identical to the one detected in the present study of 1997 (45%), again indicating a rapid spread. Subsequently, it can be assumed that the CSW network, being very mobile, has contributed to a large extent to the transmission of HIV-1 into the general population, mostly via its male clients. Finally, no other risk group–related subtype–specific HIV epidemics could be identified in Ethiopia so far, as were described in South Africa (homosexual, subtype B; heterosexual, subtype C) (34) or Thailand (intravenous drug users, subtype B; heterosexual, subtype E) (35).

**TABLE 3. Accumulation of HIV-1 diversity in samples from commercial sex workers from Addis Ababa collected at various timepoints**

<table>
<thead>
<tr>
<th>Year of sample collection</th>
<th>No. of samples</th>
<th>Mean (dS) (standard error)</th>
<th>(p) Value*</th>
<th>Mean (dN) (standard error)</th>
<th>(p) Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989/1990</td>
<td>13</td>
<td>0.1166 (0.0239)</td>
<td>—</td>
<td>0.1091 (0.0114)</td>
<td>—</td>
</tr>
<tr>
<td>1992</td>
<td>24</td>
<td>0.1465 (0.0256)</td>
<td>.25</td>
<td>0.1168 (0.0102)</td>
<td>.06</td>
</tr>
<tr>
<td>1995</td>
<td>19</td>
<td>0.1310 (0.0233)</td>
<td>.50</td>
<td>0.1407 (0.0115)</td>
<td>&lt;.05</td>
</tr>
<tr>
<td>1997</td>
<td>17</td>
<td>0.1979 (0.0328)</td>
<td>&lt;.01</td>
<td>0.1564 (0.0140)</td>
<td>&lt;.01</td>
</tr>
</tbody>
</table>

For each year of sample collection, the overall means of the proportion of synonymous substitutions per potential synonymous site (\(dS\)) and the overall means of the proportion of nonsynonymous substitutions per potential nonsynonymous site (\(dN\)) were calculated with Jukes-Cantor correction using MEGA software (39).

* \(p\) value expresses statistical significance of the difference between mean \(dS\) or mean \(dN\) values of samples collected at various time points compared with the oldest timepoint (1989/1990). These values were computed using Student's \(t\) test with infinite degrees of freedom, as previously described (39).
In this study, the WHO algorithm for HIV-1 subtyping, which includes initial screening of plasma by subtype specific V3 peptide ELISA, followed by HMA and finally DNA sequencing (36) was performed for the first time in Ethiopia. In our hands, the use of a tailor-made “Ethiopian consensus” C peptide partly increased the time in Ethiopia. In general, the peptide reactive with the WHO consensus C peptide was missed were identified, as compared to 50 of 66 (76%) by the Ethiopian C peptide. In general, the peptide sequencing, which includes initial screening of plasma by sub­

1. Primers ED3/ED14 followed by primers ED31/ED33
2. Primers ED5/ED12 followed by primers ED31/ED33

Use of ES7/8 primers is not recommended.

When the HIV-1 gp120 V3 diversity of 1997 Addis Ababa CSW samples was put in the context of diversity found in CSWs from the same city in previous years, it could be concluded that diversity increases over time. In other words, there is no evidence as yet that HIV-1 gp120 V3 diversity, within subtype C, has reached a plateau in the Addis Ababa CSW population. In addition, phylogenetic analyses performed in the present study demonstrated that there is no clustering of HIV-1 gp120 V3 sequences of CSW according to date or locale of sample collection. This indicates that the accumulation of diversity of HIV-1 gp120 V3 in Addis Ababa CSWs, although increasing, is not in a certain direction over time and in addition, that there is substantial mixing of HIV-1-positive populations in this locale.

Finally, given that this study was carried out only in Addis Ababa CSWs, further work is required to assess the existence and distribution of other subtypes in other parts of Ethiopia, especially along its borders. Knowledge about the genetic diversity and geographic distribution of HIV-1 subtypes in Ethiopia can contribute to the efficacy of future vaccine implementations.

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