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# Genetic Analysis of Human Immunodefiency Virus Type I Strains in Kenya: A Comparison Using Phylogenetic Analysis and a Combinatorial Melting Assay

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**Disciplines**
Immune System Diseases | Microbiology | Pathogenic Microbiology

**Comments**
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# Genetic Analysis of Human Immunodeficiency Virus Type 1 Strains in Kenya: A Comparison Using Phylogenetic Analysis and a Combinatorial Melting Assay

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## ABSTRACT

**We surveyed human immunodeficiency virus (HIV) subtype distribution from peripheral blood mononuclear cells (PBMCs) collected in 1995 from 24 HIV-1-infected Kenyan residents (specimens from predominantly male truck drivers and female sex workers near Mombasa and Nairobi). Processed lysates from the PBMC samples were used for *env* amplification, directly sequenced, and analyzed by phylogenetic analysis. Envelope amplification products were also used for analysis in a polymerase chain reaction (PCR)-based assay, called the combinatorial melting assay (COMA). Results of the two tests were compared for assignment of subtype for this Kenyan cohort. The COMA, a PCR capture technique with colorimetric signal detection, was used with HIV reference subtype strains as well as regional (East Africa) HIV strains for subtype identification. Performance of the COMA was at 100% concordance (24 of 24) as compared with DNA sequencing analysis. Phylogenetic analysis showed 17 isolates to be subtype A, 3 subtype D, and 4 subtype C viruses. This may represent an increase in subtype C presence in Kenya compared with previously documented reports. The COMA can offer advantages for rapid HIV-1 subtype screening of large populations, with the use of previously identified regional strains to enhance the identification of local strains. When more detailed genetic information is desired, DNA sequencing and analysis may be required.**

## INTRODUCTION

THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) global epidemic is currently thought to be composed of at least 10 phylogenetically distinct group M subtypes, designated A through J, and group O viruses.[1–4] Worldwide, and especially in African countries, where the epidemic is oldest, multiple subtypes predominate.[5,6] The surveillance and documentation of strains, both established as well as newly disseminated in countries, are important for epidemiological tracking, diagnostic testing methodologies, protection of the blood supply, vaccine development, and field trials.[7] Several techniques have been used to determine subtypes of HIV-1: (1) nucleotide sequencing,[3,4] (2) the enzyme immunoassay utilizing peptides from the variable 3 region (V3) of the gp120 region from HIV-1,[8–10] (3) the heteroduplex mobility assay (HMA),[11,12] and (4) subtype classification by polymerase chain reaction (PCR).[13] While all of these methods have particular advantages and disadvantages, nucleotide sequencing is the most definitive and informative. However, compared with sequencing, the HMA has been demonstrated to be reliable for subtyping.[14] The most frequently sequenced portion of the HIV-1 genome is the V3 and flanking regions of gp120 in the *env* gene.[4] This is due to the biological importance of this area[15–19] as well as to the large numbers of strains sequenced for this region and available for phylogenetic comparison in the Los Alamos National Laboratory HIV database.[4] Sequence analysis of this gene region has been shown to provide accurate phylogenetic relationships, using most of the commonly available methods for inferring phylogenetic relationships.[20,21] To determine the subtype distribu-

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*Note:* Use of trade names and commercial sources is for identification only and does not imply endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

tion of HIV strains in truck drivers and sex workers near Mombasa and Nairobi, Kenya, and to better understand their contribution to the dissemination and mixing of subtypes, specimens from HIV-infected persons were obtained and sequenced directly from PCR fragments from the envelope gene. Phylogenetic analysis of the sequences was compared with the results produced by a new combinatorial melting assay (COMA),[22] utilized here for HIV-1 subtype identification. The COMA subtypes an amplification product that encompasses the *env* region we sequenced, plus approximately 100 bp upstream and 100 bp downstream. The utility and performance characteristics of the two techniques are discussed.

## MATERIALS AND METHODS

### *Specimens and isolation of viral DNA*

Twenty-four HIV-1 specimens were obtained in 1995 from participants in a Kenyan sexual transmission cohort study, near Mombasa and Nairobi. The individuals were predominantly male truck drivers and female sex workers. The peripheral blood mononuclear cell (PBMC) processing and PCR amplification of the DNA lysates were as previously described,[23] except that the primary amplification primers for DNA sequencing were MK369 (5′-TGGAGCCAGTAGATCCTAGACTAGAGCCCT) and MK616 (5′-AATGGTGAGTATCCCTGCCTAACTCTATT) and the nested amplification primers for DNA sequencing were MK650 (5′-AATGTCAGCACAGTACAATGTACAC) and MK601 (5′-TTCTCCAATTGTCCCTCATATCTCCTCCTCCA). The primary amplification primers utilized for the COMA PCR product were either MK369/MK616, MK603 (5′-CAGAAAAATGGTGGGTCACAGTCTATTATGGGGTACCT) and MK602 (5′-GCCCATAGTGCTTCCTGCTGCTCCCAAGAACC), or ED5/ED12.[11] Nested amplification primers[2,22] for the COMA PCR products were LK1/LK2.[22]

### *DNA sequencing*

PCR-amplified products were purified by the Qiagen PCR purification kit (Qiagen, Chatsworth, CA). Purified DNA was sequenced according to the ABI PRISM dye terminator cycle sequencing Ready Reaction kit (Applied Biosystems/Perkin-Elmer, Foster City, CA). Sequencing primers used were various combinations of the PCR primers (MK616, MK650) and an internal sequencing primer KR207 (5′-CTGTTAAATGGCAGTCTAGC).

### *Phylogenetic analysis*

The sequences were manually aligned with the sequence editor function of the Genetic Data Environment (GDE) package[24] and positions where gaps occurred were removed from analysis, leaving 327 nucleotide sites. Percentage diversity was calculated with the pdist function of the Molecular Evolution Genetic Analysis (MEGA) program.[25] Phylogenetic analysis was accomplished with PHYLIP (Phylogeny Inference Package)[26] for both neighbor-joining and maximum likelihood trees. Pairwise evolutionary distances were estimated for the neighbor-joining trees by using the maximum likelihood option as the evolutionary model in the dnadist program. Utilizing the consense program, a consensus tree was developed from the 500 bootstrapped, neighbor-joining trees.

### *Nucleotide sequence accession numbers*

The GenBank accession numbers for the Kenyan sequences reported in this study are listed in Table 1.

### *COMA*

The COMA was performed by two of us (L.K. and S.S.) as previously described,[22] with modifications of the reference strains used. Briefly, antisense, single-stranded DNAs (ssDNAs) were generated for reference subtypes A, B, C, and D by asymmetrical PCR, with biotinylation of one of the primers facilitating capture to streptavidin-coated plates. Reference and regional strains used for the different subtypes (followed by each GenBank accession number) were as follows: A (92KEML1048:AF103913, 91KEMCH9727:AF103910, 91KEMCHI96:AF103911, and 92KEML639:AF103914), B (BR20:U08797 and TH14:U08801), C (SM145:L22946, DJ259:L22940, and MW6510:L15733), and D (91KEML22:AF10312, 89KEML203:AF103909, UG21:U27399, and UG46:U08809). Reference PCR products from the same subtype were combined to form reference pools and placed in rows of the microtiter plate. Complementary sense-stranded molecules from the Kenyan DNA were produced by asymmetrical PCR with digoxigenin labeling. These samples were placed in the microtiter plate columns, allowing heteroduplex formation between the Kenyan DNA and reference subtype DNA. The subtype was determined by a colorimetric immunoassay using alkaline phosphatase-conjugated antibody against digoxigenin. The correct subtype was determined by the comparative absorbance measurement, rounded to the first decimal place, being higher than any other by a factor of at least two.

## RESULTS

### *PCR amplification and nucleotide analysis*

PCR amplification of the C2V3 region of the *env* gene was performed with two sets of primers in a nested fashion in two separate PCRs. Nucleotide sequences were obtained directly from the PCR product and aligned for homologous positions, and a neighbor-joining phylogenetic tree was constructed (Fig. 1). Seventeen of the Kenyan sequences clustered within the subtype A group, 4 with subtype C, and 3 with subtype D. The same subtype classifications were obtained with a tree constructed with the maximum likelihood program (data not shown). Two pairs of sequences (KEML1/KEQRS1, KETK5/KEML4) clustered together with bootstrap support greater than 70% (Fig. 1) as well as in the maximum likelihood tree. These sequences are from female sex workers and a female sex worker/male truck driver, respectively (see caption to Fig. 1). Whether these pairs represent epidemiologically linked infections is unknown, yet the sequences within each pair being derived from individuals residing in different cities (Mombasa and Nairobi) suggests some infection links between these commu-

TABLE 1. COMA VERSUS SEQUENCING SUBTYPING RESULTS OF THE STUDY SPECIMENS

| *Isolate* | *GenBank accession numbers* | *Relative absorbance densities (490 nm) of DNA heteroduplexes formed by complementary ssDNA from each unknown* env *and four reference* env *repertoires* | | | | *Subtype determined by COMA* | *Subtype determined by DNA sequencing* |
|---|---|---|---|---|---|---|---|
| | | *A* | *B* | *C* | *D* | | |
| KEMCH1 | AF103916 | **1.00** | 0.12 | 0.10 | 0.13 | A | A |
| KEMCH3 | AF103917 | **1.00** | 0.09 | 0.13 | 0.11 | A | A |
| KEMCH4 | AF103918 | **1.00** | 0.13 | 0.13 | 0.13 | A | A |
| KEML3 | AF103921 | **1.00** | 0.08 | 0.09 | 0.10 | A | A |
| KEML4 | AF103922 | **1.00** | 0.08 | 0.08 | 0.08 | A | A |
| KEML5 | AF103923 | **1.00** | 0.17 | 0.17 | 0.21 | A | A |
| KEML6 | AF103924 | **1.00** | 0.20 | 0.13 | 0.48 | A | A |
| KEQRS2 | AF103926 | **1.00** | 0.17 | 0.22 | 0.24 | A | A |
| KEQRS4 | AF103928 | **1.00** | 0.11 | 0.10 | 0.15 | A | A |
| KETDP1 | AF103930 | **1.00** | 0.12 | 0.14 | 0.18 | A | A |
| KETDP2 | AF103931 | **1.00** | 0.11 | 0.13 | 0.13 | A | A |
| KETK2 | AF103934 | **1.00** | 0.07 | 0.11 | 0.10 | A | A |
| KETK3 | AF103935 | **1.00** | 0.11 | 0.12 | 0.12 | A | A |
| KETK4 | AF103936 | **1.00** | 0.10 | 0.16 | 0.14 | A | A |
| KETK5 | AF103937 | **1.00** | 0.07 | 0.17 | 0.08 | A | A |
| KESHH1 | AF103929 | **1.00** | 0.07 | 0.04 | 0.11 | A | A |
| KEX1 | AF103938 | **1.00** | 0.07 | 0.09 | 0.10 | A | A |
| KEML1 | AF103919 | 0.27 | 0.12 | **1.00** | 0.18 | C | C |
| KEQRS1 | AF103925 | 0.28 | 0.28 | **1.00** | 0.23 | C | C |
| KETDP3 | AF103932 | 0.19 | 0.26 | **1.00** | 0.18 | C | C |
| KETK1 | AF103933 | 0.51 | 0.07 | **1.00** | 0.24 | C | C |
| KEMCH2 | AF103916 | 0.26 | 0.46 | 0.11 | **1.00** | D | D |
| KEML2 | AF103920 | 0.09 | 0.08 | 0.11 | **1.00** | D | D |
| KEQRS3 | AF103927 | 0.11 | 0.08 | 0.12 | **1.00** | D | D |

nities. Subtypes A and C are represented in these two pairs, and all three subtypes found in this study have previously been reported in Kenya.[6,27,28]

The percentage diversity of the nucleotide sequences was calculated for the Kenyan taxa in Fig. 1. The mean genetic distance among Kenyan subtype A sequences is 13.2%, while the subtype C distance is 11.2% and the subtype D distance is 10.7%. An alignment of the predicted amino acid sequences for the 24 Kenyan sequences is depicted in Fig. 2. All are compared with subtype-specific consensus sequences from the Los Alamos HIV database.[4]

The GPGQ tetrameric tip of the V3 loop was predominant in all three subtypes, with the exception of two type As (KESHH1, KEX1) and one type D (KEQRS3) having GPGR. Positively charged amino acids at positions 11 and 25 of the V3 loop, generally believed to be associated with a syncytium-inducing phenotype,[29] were absent in all of the Kenya sequences. This would be expected for subtype A strains, but positively charged amino acids at one or two of these positions usually make up the majority of published African type D strains.[6,14,30] One explanation could be that these Kenyan strains may be from individuals in an early stage of infection,[31,32] although clinical data are not available.

### *COMA and comparison with sequencing*

PCR amplification of the C2V4 region of the *env* gene was performed with one set of primers and subsequently sent to two of us (L.K. and S.S.)[22] for secondary amplification and COMA testing (see Materials and Methods). Table 1 lists the COMA results by relative absorbance densities of the 24 Kenyan specimens in comparison with the subtype determined by sequencing and phylogenetic analysis. All specimens are in agreement between the two methods, corresponding to a 100% concordance (24 of 24).

## DISCUSSION

### *COMA versus sequencing analysis for HIV-1 subtype identification*

Along with investigating the distribution of strains among the highly mobile cohort of truck drivers and sex workers, we compared the accuracy of the COMA with DNA sequencing and subsequent phylogenetic analysis and adapted the COMA in a region-specific fashion for subtyping HIV strains from Kenya. Phylogenetic analysis was performed on a genomic region within and approximately 200 bp shorter than the PCR fragment used in the COMA. The extensive use of this region by us and others for accurate subtyping allowed the utility of sequencing this shorter fragment for comparisons with the COMA results. The complete concordance for this Kenyan HIV-1 sampling between the two methodologies (100%, 24 of 24) demonstrated that the two *env* regions could be compared reliably by these methods (although the sample size is relatively

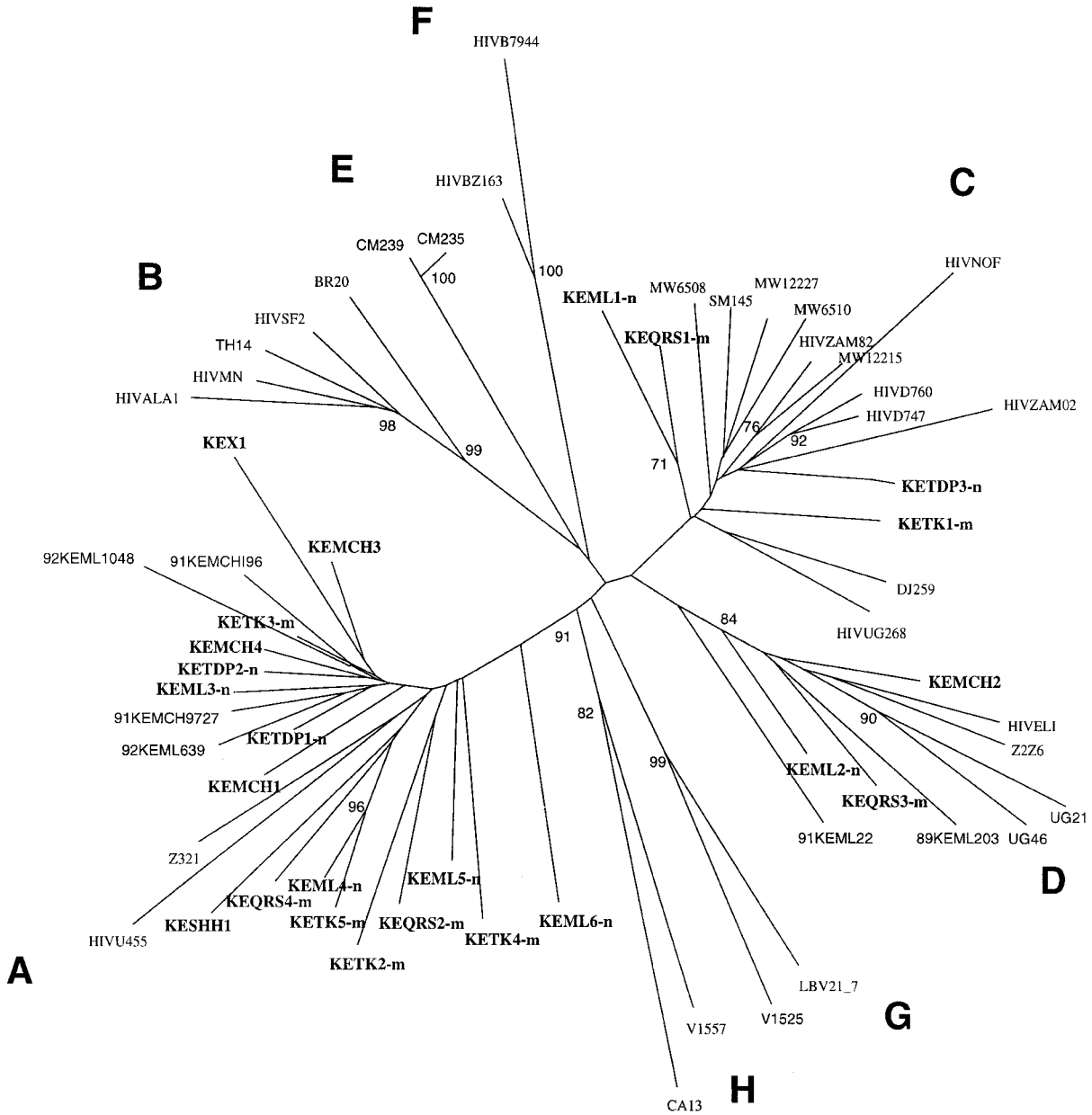


**FIG. 1.** Phylogenetic analysis of 24 HIV-1 DNA specimens obtained from individuals in Kenya. Analysis is based on 327 alignable positions (positions in alignment containing one or more gaps are excluded from analysis) in the C2V3 region of the HIV-1 *env* gene with the simian immunodeficiency virus $SIV_{cpzgab}$ as outgroup. Los Alamos HIV Database subtype sequences from A, B, C, D, E, F, G, and H are included to facilitate subtype clustering of the Kenyan sequences. The Kenyan sequences determined in this study (boldface with a KE prefix, isolation date of 1995) are designated as follows: MCH, female sex workers—region unknown; ML, female sex workers from Nairobi; QRS, female sex workers from Mombasa; TDP, male truck drivers near Nairobi; TK, male truck drivers near Mombasa; SHH, male sexually transmitted disease patient; X, unknown. For clarity, those originating from Mombasa have an -m suffix and those from Nairobi have an -n suffix. The numbers at the branch points indicate the percentage of times the branch was preserved in the 500 bootstrap replications. Only bootstrap proportions >70% are shown. The large letters indicate A–H subtypes.


**FIG. 2.** Amino acid alignment of the *env* C2V3 regions of the Kenyan strains compared with subtype A, C, and D consensus sequences.[20] Kenyan descriptive names are as listed in the caption to Fig. 1. A dash (–) indicates identity with the A, C, or D consensus sequence and a period (.) depicts a deletion.

small) and supports the use of the COMA to subtype previously unknown samples. A key feature of the COMA is its flexible use of multiple strains from each subtype for detection of the unknown strain. Utilization of strains found in the geographic region of interest as reference strains is a customization of the more general COMA, which is run with worldwide reference strains for subtyping.[22] Specimens from subtypes A and D, which have been reported to predominate in Kenya and Uganda, were used in the cocktail of reference strains. The Kenyan reference strains were from female sex workers, isolated from 1989 to 1992. Further refinement of signal-to-noise ratios for the COMA may be assisted by addition of more recently subtyped specimens to the respective subtype reference pool. As we demonstrated here, a population-based study could be performed using the COMA technology for initial subtype identification, without DNA sequencing. Ambiguous or cross-reacting specimens (i.e., subtype recombinants or dual infections) could then be sequenced for further identification, if needed.

In addition, the COMA can be used to systematically monitor change in subtype distribution over time in large population-based surveys. Immunology-based assays such as peptide-binding enzyme immunoassays (EIAs) have been effective where the epidemic was relatively new and, consequently, the diversity within subtypes was low, e.g., in Thailand.[9] In regions where the epidemic is older and people have been infected for longer periods, the with-in person distribution of quasispecies is more complex, and the host immune system has responded to many antigenically distinct variants. These samples typically have a large degree of cross-reactivity in serological peptide-based assays. Therefore, the HMA[11,14] and COMA techniques, as demonstrated previously[22] and in this study, are more reliable for subtype assignment. Subtype analysis with the HMA typically uses two reference strains per subtype, while the microtiter format of the COMA allows many reference strains to be used with no increase in the number of wells. Sequencing and subsequent phylogenetic analysis, although more time consuming, is generally considered the "gold standard." The COMA has the advantages of using a format typical of that widely used in EIA technology, requiring little equipment beyond a thermocycler for PCR, and being easy to interpret. The HMA could be used as a complement to initial COMA screening by identifying major and minor quasispecies of samples that yield indeterminate COMA results. Alternatively, or as the next step, the PCR-amplified specimen could be sequenced for definitive typing, mutation analysis, amino acid sequence prediction, and identification of recombinant viruses.

### *Kenyan HIV-1 subtypes*

HIV-1 subtyping has been an important part of the surveillance and characterization of HIV strains for designing vaccine strategies prior to conducting vaccine field trials. Many African countries harbor a large variety of subtypes represented in the global epidemic. In particular, the Central and East African countries, which includes Kenya, compose that region of the continent where most of the subtypes have been reported.[14,33] Our interest in tracking the spread of HIV-1 subtypes in this area led us to obtain and analyze 24 specimens from a Kenyan sex worker/truck driver cohort. The finding that most (17 of 24) of these strains grouped with subtype A and a minority with subtype D (3 of 24) is similar to previously published reports of Kenyan sequences[6] and is also consistent with the rapid spread of subtype A in Central Africa.[14] Subtype C (4 of 24 in this study) is also well established in several southern African countries,[7,34,35] as well as in India.[36] The spread of this subtype to other countries includes China,[37] Malaysia,[38] Russia,[39] England,[40] and the United States (P. Sullivan, Centers for Disease Control [CDC, Atlanta, GA], unpublished data, 1998). The United Nations Program on AIDS (UNAIDS, Geneva, Switzerland) has identified subtypes A and C as the most prevalent

group M strains worldwide and has organized collaborative international studies for better sampling and characterization of these subtypes.[41] The identification of the C subtype in these Kenyan residents is further documentation of its continuing spread. Previous Kenyan HIV subtype sampling studies[6,27,28] for specimens collected from 1991 to 1994 had demonstrated only one subtype C strain. Although the sampling numbers are relatively small, the increased number of subtype C virus strains identified here could indicate an increasing prevalence of subtype C HIV strains in Kenya. It at least establishes that the initial report[28] of a subtype C virus in Kenya was not spurious and that subtype C viruses are being transmitted in this region.

The worldwide trend toward an increase in multiple subtypes in regions where only a few previously predominated is typically associated with increased travel and demographic shifts.[42] Truck drivers involved in this study transport goods along the trans-African highway, beginning in Mombasa (a major regional seaport), with final destinations in Uganda, Tanzania, Rwanda, Burundi, eastern Zaire, and southern Sudan.[43] The drivers are on the road for many weeks or months and have frequent contact with female sex workers along the route. Sex workers are also mobile along the truck route, sometimes accompanying drivers. Both drivers and sex workers have high rates of HIV-1 infection. Thus, these individuals may be exposed to HIV-1 subtypes from a wide geographic area and could provide a means of introducing new HIV-1 subtypes (subtype C in this case) into new geographic areas. The observation that two (A and C) of the three subtypes found here have phylogenetically linked sequences from residents of different Kenyan cities may further indicate the effectiveness of spread by the high-risk behavior within these risk groups.

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