Genotypic Variability of HIV-1 *Reverse Transcriptase* Gene from Long-Term Antiretroviral-Experienced Patients in Kenya

Timothy J. Nzomo,¹⁻³ Rose C. Kitawi,¹⁻³ Ruth S. Mwatelah,¹⁻³ Rashid Aman,^{1,4,5}
Maureen J. Kimulwo,¹⁻³ Geoffrey Masankwa,^{1,3} Javan Okendo,⁶ Raphael M. Lwembe,⁷
Bernhards Ogutu,^{1,5,7} Anne Muigai,³ and Washington Ochieng^{1,7,8}

Abstract

There is continuous need to track genetic profiles of HIV strains circulating in different geographic settings to hasten vaccine discovery and inform public health and intervention policies. We partially sequenced the reverse transcriptase region of the HIV-1 *pol* gene from a total of 54 Kenyan patients aged 18–56 years who continued highly active antiretroviral treatment (HAART) for between 8 and 102 months. Subtyping was done using both the JPHMM tool and phylogenetic method. HIV-1 subtype A1 was the predominant strain in circulation, representing 57.4% and 70.4% of all isolates as determined by JPHMM and phylogenetic methods, respectively. Subtypes D (14.8%, 7.4%), C (5.6%, 9.3%), and A2 (0%, 5.6%) were determined at respective prevalence by both methods. JPHMM identified 22.2% of the isolates as recombinants. This surveillance focused on the *RT* gene and reaffirms the predominance of subtype A and an increasing proportion of recombinant strains in the Kenyan epidemic.

THE MAJORITY OF THE GLOBAL HIV-1 epidemic is represented by genetically diverse forms of the virus.^{1,2} The evolutionary rate of HIV has been shown to track with epidemic events or milestones, including rollout of highly active antiretroviral treatment (HAART) and changes in prevalence.³ Of the global epidemic, subtype C contributes about 50% of all infections, while recombinants account for approximately 20%. HIV-1A and B account for about 12%, 11%, and 20% of the global HIV epidemic, respectively, with subtype B being restricted to Europe, North America, and parts of North Africa. In Africa, HIV infection exhibits distinct regional trends with varying rates and prevalence.⁴

The Kenyan epidemic has declined significantly and steadily over the past 8 years to stabilize at low levels of 5.6%,^{5,6} although there are key demographic pockets for escalating new infection.⁷ The overall improvements in HIV prevalence in Kenya can be attributed to gains in uptake of testing and scaled access to HAART.⁸ But increased access to HAART presents special challenges in diagnosing new in-

fections, monitoring of the epidemic, and managing subepidemics as new or mutant virus strains appear in circulation.⁹

We recently reported on HIV subtype variability in Kenyan HAART patients based on envelope sequences.¹⁰ The epidemiologic architecture of the Kenyan epidemic displays characteristic variability at both genetic and geographic levels, with HIV-A dominating, although at a declining prevalence.^{10–13} Here, we report the genetic diversity of the HIV-1 *RT* gene among patients who have sustained HAART for between 8 and 102 months.

Fifty-four patients aged 18 to 56 years were enrolled between February and September 2013 from six comprehensive care centers located in Kiambu, Kajiado, Naivasha, Malindi, Kisumu, and Homa Bay counties. Five milliliters of EDTA venous blood was drawn from each patient and fractionated to obtain peripheral blood mononuclear cells (PBMCs) and plasma as described earlier.¹⁰ RNA was extracted from plasma of patients with viral load (VL) above 1,000 RNA copies/ml using the QiaAmp Viral RNA Mini Kit while DNA

¹Center for Research in Therapeutic Sciences, Strathmore University, Nairobi, Kenya.

²Institute of Tropical Medicine and Infectious Diseases, Nairobi, Kenya.

³Jomo Kenyatta University of Agriculture and Technology, Nairobi, Kenya.

⁴African Center for Clinical Trials, Nairobi, Kenya.

⁵Institute of Healthcare Management, Strathmore University, Nairobi, Kenya.

⁶Kenyatta University, Nairobi, Kenya.

⁷Kenya Medical Research Institute, Nairobi, Kenya.

⁸Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts.

was isolated from PBMCs of patients with VL up to 1,000 HIV RNA copies using the QiaAmp DNA Mini Kit and according to manufacturer's instructions. For the RNA, a 701-base pair segment of the HIV-1 *pol-RT* gene corresponding to nucleotides 2480–3180 of HIV-1_{HXB2} was amplified in a One-Step reverse transcriptase polymerase chain reaction (RT-PCR) protocol (Qiagen) followed by a nested PCR. Primers RT18 (5'-GGAAACCAAAAATGATAGGGGGAA TTGGAGG-3') and KS104 (5'-TGA CT T G C C C A A T T T A G T T T T C C C A C T A A -3') were used, respectively, in the forward and reverse RT-PCR reactions. A final PCR reaction volume of 25 μ l included a 1 $\times$ PCR buffer, 0.4 mM dNTPs, 1.5 mM MgCl₂, 5 μ l of RNA template, 1 μ l enzyme mix, 0.6 μ M of each primer, and 5 units of RNaseOut. Reverse transcription was performed at 50°C for 30 min followed by amplification steps of 15 min denaturation at 95°C and 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 10 min.

Second round PCR was done using HotStar taq polymerase (New England Biolabs) in a 25 μ l reaction volume comprising 1 $\times$ PCR buffer, 0.2 mM dNTPs, 2 mM MgCl₂, 0.5 μ M forward and reverse primers, 0.625 units of the enzyme, and 3 μ l of RT-PCR product as template. The thermocycling profile comprised denaturation at 95°C for 5 min followed by 40 PCR cycles of 95°C denaturation for 30 s, annealing at 56°C for 45 s, and extension at 68°C for 45 s. A final extension was done at 68°C for 10 min. KS101 (5'-GTAGGACCTAC ACCTGTTCAACATAATTGGAAG-3') and KS102 (5'-CC CATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3') were used as forward and reverse nested PCR primers, respectively. When DNA was used as the starting material, PCR procedures were similar except that the RT step was excluded. The PCR products were purified with Qiaquick purification columns and sequenced using standard Big-Dye chain terminator chemistry.

Sequences were manually inspected, joined in pairwise alignment (BioEdit ver. 7.2.5), and subjected to multiple sequence alignment using Clustal W (Supplementary Data; Supplementary Data are available online at www.liebertpub.com/aid). Two reference sequences were included in the alignment for each subtype query sequence. Phylogenetic analyses were done using the neighbor-joining method with 1,000 bootstraps. Evolutionary distances were computed using the Kimura two-parameter method, with gaps partially deleted at 50% cutoff. Phylogenetic trees were generated using MEGA version 6.06 and used to assign subtypes (Fig. 1). Alternate subtype assignment was done using the JPHMM tool for comparison. All the sequences reported in this article have been deposited and accessioned at the GenBank. Ethical approval was granted by the KEMRI Scientific and Ethics

FIG. 1. Phylogenetic tree of HIV *pol-RT* sequences from 54 highly active antiretroviral treatment (HAART) patients. The *RT* gene was partially sequenced using primers amplifying a 701-base pair region. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed and all positions with less than 50% site coverage were eliminated. Node statistics corresponding to less than 45% bootstrap values are hidden to minimize clutter. Reference sequences are italicized and shown by filled diamonds. Scale bar = 0.02.

TABLE 1. HIV SUBTYPE, PATIENT AND TREATMENT INFORMATION OF STUDY SUBJECTS

Patient ID	GenBank accession number	Age	Sex	HAART regimen	Subtype
HND200	KM853096	42	F	NR	A1
KAH004	KM853097	24	F	TDF/3TC/NVP	N.C
KAH010	KM853098	34	F	TDF/3TC/NVP	A1
KHC106	KM853099	35	F	d4T/3TC/NVP	A1
KHC160	KM853100	25	F	TDF/3TC/NVP	A1
KHC163	KM853101	26	F	AZT/3TC/NVP	A1
KMB145	KM853102	38	F	TDF/3TC/NVP	A1
KMB160	KM853103	43	M	AZT/3TC/NVP	A1
KMB077	KM853104	45	M	TDF/3TC/NVP	N.C
MLD245	KM853105	32	F	AZT/3TC/NVP	A2
MLD011	KM853106	31	F	NR	D
MLD183	KM853107	7	F	ABC/3TC/NVP	A1
MLD191	KM853108	34	M	TDF/3TC/NVP	D
MLD198	KM853109	26	F	d4T/3TC/NVP	A1
MLD021	KM853110	41	M	TDF/3TC/NVP	D
MLD040	KM853111	49	F	NR	A1
MLD541	KM853112	53	M	d4T/3TC/NVP	A1
MLD545	KM853113	52	F	AZT/3TC/EFV	A1
MLD548	KM853114	46	F	d4T/3TC/NVP	N.C
MLD060	KM853115	36	F	TDF/3TC/EFV	C
KHC093	KM853116	43	M	d4T/3TC/NVP	A1
NVS058	KM853117	30	F	AZT/3TC/NVP	A1
MLD001	KM853118	30	M	AZT/3TC/NVP	A1
MLD002	KM853119	29	F	AZT/3TC/NVP	A2
MLD003	KM853120	44	F	AZT/3TC/EFV	A1
MLD008	KM853121	45	M	d4T/3TC/NVP	A1
MLD009	KM853122	47	M	AZT/3TC/NVP	A1
MLD010	KM853123	22	F	None	C
MLDU011 ^a	KM853124	46	F	TDF/3TC/NVP	D
MLD012	KM853125	56	F	d4T/3TC/NVP	A1
MLD013	KM853126	39	M	d4T/3TC/NVP	A1
MLD014	KM853127	50	M	None	A1
MLD016	KM853128	46	F	NR	A1
MLD166	KM853129	28	M	d4T/3TC/NVP	A1
MLD167	KM853130	32	F	d4T/3TC/NVP	C
KHC016	KM853131	28	M	TDF/3TC/NVP	A1
KHC025	KM853132	32	F	AZT/3TC/NVP	D
KHC040	KM853133	29	F	TDF/3TC/NVP	C
KHC045	KM853134	31	F	AZT/3TC/NVP	A1
KHC048	KM853135	22	F	d4T/3TC/NVP	A1
KAH016	KM853136	49	M	TDF/3TC/NVP	A1
KMB039	KM853137	35	F	TDF/3TC/NVP	A2
KMB067	KM853138	50	M	d4T/3TC/NVP	A1
MLD185	KM853139	52	M	AZT/3TC/NVP	A1
NVS090	KM853140	46	F	AZT/3TC/NVP	A1
NVS095	KM853141	28	M	d4T/3TC/NVP	A1
NVS032	KM853142	47	M	AZT/3TC/NVP	A1
NVS047	KM853143	39	F	d4T/3TC/EFV	A1
NVS052	KM853144	45	M	AZT/3TC/EFV	A1
NVS056	KM853145	56	F	d4T/3TC/EFV	A1
NVS069	KM853146	41	M	d4T + 3TC + NVP	A1
NVS085	KM853147	37	M	TDF/3TC/NVP	A1
NVS122	KM853148	28	F	d4T/3TC/NVP	A1
NVS152	KM853149	30	F	AZT/3TC/EFV	C

^aMLDU011 is an injecting drug use subject and is unique from MLD011.

NR, not reported; HAART, highly active antiretroviral therapy; N.C, not classified—sequence did not cluster discretely with any of the reference sequences; TDF, tenofovir; 3TC, lamivudine; NVP, nevirapine; d4T, stavudine; AZT, zidovudine; ABC, abacavir; EFV, efavirenz.

Review Unit protocol #2477. Corresponding patient and genotypic data are provided in Table 1. Using the JPHMM subtyping tool, 57.4% of the 54 sequences were determined to be subtype A1 while subtypes C, D, and recombinants were 5.6%, 14.8%, and 22.2%, respectively. The majority ($n=6$, or 50%) of the RT recombinants were AC (A1C, $n=5$ and A2C, $n=1$). Others included A1A2 ($n=2$) and A1D, A2B, A2D, and CD ($n=1$, each). No isolate was identified as pure subtype A2 by this method.

The phylogenetic method assigned more viruses as subtype A (76%), with the majority being A1 (70.4%). Seven of the 38 subtype A1 isolates were classified as A1A2 ($n=2$) or A1C ($n=5$) recombinants by JPHMM. The rest of the subtypes based on phylogenetics were HIV-1C at 9.3% and HIV-1D at 9.3%. Two sequences, MAD021 and MLD185, were substantially shorter than the rest, running just above half the length of the amplified RT gene segment. These isolates were identified by both methods as subtype D and A1, respectively, and are thus deemed to be accurately classified. Three isolates, KMB077, KAH004, and MLD548, that were determined as subtypes D, A1D, and D by JPHMM, respectively, did not cluster distinctly with any reference sequences on the phylogenetic tree and were “not classified” by this method.

Six (or 50%) of the recombinants were from the coastal region of Malindi. Of the 54 sequences, 27.8% contained reverse transcriptase inhibitor (RTI) drug resistance-associated mutations (DRAMs). Seventy-three percent of these mutations conferred resistance to both nucleoside RTIs (NRTIs) and nonnucleoside RTIs (NNRTIs), with the common ones being M184V/I and K103N, respectively. A few studies have recently endeavored to highlight differences in efficiency of different genotyping tools used for HIV subtyping.^{14,15} We recently showed slight but useful differences in detecting HIV-1 subtypes based on the envelope C2V3 gene when using both the JPHMM and phylogeny.¹⁰ This article reaffirms the same observations using the *pol-RT* gene, with both JPHMM and phylogenetic methods concurring on subtype assignment in 72.2% (39/54) of the isolates.

While subtype A HIV-1 is still predominant in Kenya, recombinant viruses may be on the rise. Whereas the phylogenetic method is robust at classifying HV strains based on evolutionary distances, it is not powered to detect recombinant viruses. Hence efficient detection tools should be developed or existing methods harnessed to sensitively identify small genetic differences that might influence the overall molecular pattern of the circulating epidemic. Such efforts will empower routine genetic surveillance to better inform public health and vaccine or drug discovery efforts.

GenBank Accession Numbers

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Author Disclosure Statement

No competing financial interests exist.

References

1. Aldrich C and Hemelaar J: Global HIV-1 diversity surveillance. *Trends Mol Med* 2012;18(12):691–694.
2. Hemelaar J, Gouws E, Ghys PD, and Osmanov S: Global trends in molecular epidemiology of HIV-1 during 2000–2007. *AIDS* 2011;25(5):679–689.
3. Maljkovic Berry I, Athreya G, Kothari M, *et al.*: The evolutionary rate dynamically tracks changes in HIV-1 epidemics: Application of a simple method for optimizing the evolutionary rate in phylogenetic trees with longitudinal data. *Epidemics* 2009;1(4):230–239.
4. Kamali A, Price MA, Lakhi S, *et al.*: Creating an African HIV Clinical Research and Prevention Trials Network: HIV Prevalence, Incidence and Transmission. *PLoS One* 2015;10(1): e0116100.
5. Kimanga DO, Ogola S, Umuro M, *et al.*: Prevalence and incidence of HIV infection, trends, and risk factors among persons aged 15–64 years in Kenya: Results from a nationally representative study. *J Acquir Immune Defic Syndr* 2014;66:S13–S26.
6. Waruiru W, Kim AA, Kimanga DO, *et al.*: The Kenya AIDS indicator survey 2012: Rationale, methods, description of participants, and response rates. *J Acquir Immune Defic Syndr* 2014;66:S3–S12.
7. Price MA, Rida W, Mwangome M, *et al.*: Identifying at-risk populations in Kenya and South Africa: HIV incidence in cohorts of men who report sex with men, sex workers, and youth. *J Acquir Immune Defic Syndr* 2012;59(2):185–193.
8. Grabbe KL, Menzies N, Taegtmeier M, *et al.*: Increasing access to HIV counseling and testing through mobile services in Kenya: Strategies, utilization, and cost-effectiveness. *J Acquir Immune Defic Syndr* 2010;54(3):317–323.
9. Mee P, Collinson MA, Madhavan S, *et al.*: Evidence for localised HIV related micro-epidemics associated with the decentralised provision of antiretroviral treatment in rural South Africa: A spatio-temporal analysis of changing mortality patterns (2007–2010). *J Global Health* 2014;4(1):010403.
10. Kitawi RC, Nzomo TJ, Mwatelah RS, *et al.*: HIV-1 subtype diversity based on envelope C2V3 sequences from Kenyan patients on highly active antiretroviral therapy. *AIDS Res Hum Retroviruses* 2015;31(4):452–455.
11. Khamadi SA, Lihana RW, Mwaniki DL, *et al.*: HIV type 1 genetic diversity in Moyale, Mandera, and Turkana based on env-C2-V3 sequences. *AIDS Res Hum Retroviruses* 2008;24(12):1561–1564.
12. Khamadi SA, Lihana RW, Osman S, *et al.*: Genetic diversity of HIV type 1 along the coastal strip of Kenya. *AIDS Res Hum Retroviruses* 2009;25(9):919–923.
13. Khamadi SA, Ochieng W, Lihana RW, *et al.*: HIV type 1 subtypes in circulation in northern Kenya. *AIDS Res Hum Retroviruses* 2005;21(9):810–814.
14. Yebra G, de Mulder M, Martin L, *et al.*: Sensitivity of seven HIV subtyping tools differs among subtypes/recombinants in the Spanish cohort of naive HIV-infected patients (CoRIS). *Antiviral Res* 2011;89(1):19–25.
15. Pineda-Pena AC, Faria NR, Imbrechts S, *et al.*: Automated subtyping of HIV-1 genetic sequences for clinical and surveillance purposes: Performance evaluation of the new REGA version 3 and seven other tools. *Infect Genet Evol: J Mol Epidemiol Evolution Genet Infect Dis* 2013; 19:337–348.

Address correspondence to:

Washington Ochieng
Center for Research in Therapeutic Sciences
Strathmore University
P.O. Box 59857-00200
Nairobi
Kenya

E-mail: ochiengwashington@gmail.com
wochieng@strathmore.edu
wochieng@kemri.org