Molecular Epidemiology of HIV Type 1 Subtypes in Rwanda

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Abstract

HIV-1 infection is characterized by genetic diversity, with multiple subtypes and recombinant variants circulating, particularly in sub-Saharan Africa. During the Rwandan genocide, many women experienced multiple rapes and some became HIV-1 infected. We studied plasma and peripheral blood mononuclear cells (PBMCs) from 30 infected women comprising two exposure groups: those with numerous contacts, raped multiple times, and women with one lifetime sexual partner and no history of rape. Population-based sequences from *gag*, *pol*, and *env* genes were analyzed to determine HIV-1 subtypes and intersubtype recombination. Individual plasma-derived variants from 12 women were also analyzed. Subtype A was found in 24/30 (80%), intersubtype recombination (AC and AD) in 4/30 (13%), and subtypes C and D in 1/30 each. In two subjects, the pattern of HIV-1 recombination differed between plasma and PBMC-derived sequences. Intersubtype recombination was common, although there were no significant differences in subtype or recombination rates between exposure groups.

HUMAN IMMUNODEFICIENCY VIRUS type 1 (HIV-1) infection is characterized by genetic diversity with multiple subtypes and recombinant variants driving the epidemic. During the Rwandan genocide of 1994, many women experienced multiple rapes during a short time period and some became HIV-1 infected.^{1,2} These women were subjected to multiple, traumatic events involving potential HIV-1 exposure from multiple men, and thereby were at high risk of exposure to multiple HIV-1 variants or subtypes. Studying the effect of multiple, traumatic sexual exposure during a short time period is relevant to the understanding of HIV-1 pathogenesis and viral evolution. Previous studies of HIV-1 subtypes and recombination in African women have focused on female sex workers, but not victims of multiple, traumatic sexual exposures, as occur in conflict zones.³ Furthermore, investigation of the molecular epidemiology of HIV-1 in Rwanda, using more recent samples, provides the current HIV-1 subtype distributions and rates of intersubtype recombination.

The Rwandan Women's Interassociation Study and Assessment (RWISA) is a longitudinal cohort study of HIV-1-

infected women; over 50% of the cohort consisted of survivors of genocidal rape. The cohort maintains a repository of specimens collected prior to initiating antiretroviral therapy (ART). This cohort provides a rare opportunity to study the relationship between multiple traumatic sexual exposures and the presence of dual HIV-1 infections and intersubtype recombination. Recombination within a subtype (intrasubtype) occurs frequently, but it is difficult to identify it because the sequences are too similar to allow detection. We have previously reported intrasubtype recombination in subtype B viruses by analyzing sequences from the blood and female genital tract compartments.⁴⁻⁷ Intersubtype recombination is easier to detect because the sequences are genetically distinct.

At the end of 2011, 1,551 sequences were reported from Rwanda and were available in the Los Alamos HIV database (www.hiv.lanl.gov). The overwhelming majority, 95%, were subtype A. Only 2.5% were subtype C and the remaining 2.5% were subtype D or intersubtype recombinants. However, the majority of the reports are based on the analyses of short sequences.^{8,9} A handful of near full-length and fairly large

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genome sequences were reported from Rwandan patients including an A/C recombinant virus.¹⁰ These sequences were obtained from samples collected in 1992, before the genocide.

In this study, we present the current HIV-1 subtype distributions, intersubtype recombination rates, HIV-1 tropism, and drug resistance analyses from 30 HIV-1-infected Rwandan women who were enrolled in the RWISA cohort (Table 1). The women were divided into two groups based on their HIV exposure history. The first group, with numerous contacts, included 11 women who were raped multiple times during the 1994 genocide and were presumed to be infected at that time. As a consequence of the rapes they may have been exposed to multiple viral strains and subtypes. The second group, with one contact, included the remaining 19 women who reported having a single lifetime consensual sexual partner, and no history of rape. The study was approved by the Rwandan government Ethics Committee and the Institutional Review Board of the Montefiore Medical Center, Bronx, NY, and the New York State Department of Health, Albany, NY. Each participant provided informed consent.

Total RNA was extracted by using NucliSENS easyMAG bioMérieux, Inc. (Durham, NC) following the manufacturer's instructions. Briefly, total RNA was extracted using 0.5 ml plasma and the purified RNA was eluted with 25 μ l elution buffer and 5 μ l of the RNA was used for reverse transcriptase poly-

merase chain reaction (RT-PCR) amplifications as previously described.^{7,11} The SuperScript III First-Strand Synthesis System kit (Invitrogen, Carlsbad, CA) was used to synthesize first strand cDNA using 5 μ l of the purified total RNA following the manufacturer's instructions in a 20 μ l final volume. After the completion of first-strand synthesis or RT-PCR, excess RNA template was removed from the cDNA:RNA hybrid molecule by RNase H (2 U/ μ l) digestion. The cDNA was either immediately used for PCR amplification or stored at -80°C for future use.

The *gag*, *pol*, and *env* gp120 genes were PCR amplified using the GeneAmp XL PCR Kit and rTth DNA polymerase enzyme (Applied Biosystems, Foster City, CA) and hot start PCR as previously described.¹² The rTth DNA polymerase has an efficient DNA synthesis and proofreading capability, reducing PCR-mediated recombination. To amplify each gene fragment, we used a nested PCR approach using outer and inner primer pairs as previously described.^{4,5,7,12} Sequences of primers used in this study are listed in Table 2.

HIV-1 genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) by using the DNeasy blood and tissue kit (QIAGEN, Valencia, CA) following the manufacturer's instructions. We amplified HIV-1 *gag* and *pol* proviral DNA by using AccuPrime Taq DNA polymerase high fidelity enzyme (Life Technologies, Carlsbad, CA) following the manufacturer's recommendations.

TABLE 1. SUMMARY OF PATIENT CHARACTERISTICS AND HIV-1 SUBTYPE RESULTS

No.	Patient ID	Risk group	Age	CD4 ⁺ T cell count	Viral load log copies/ml	Plasma gene-specific subtypes			PBMC gene-specific subtypes	
						<i>gag</i>	<i>pol</i>	<i>gp120</i>	<i>gag</i>	<i>pol</i>
1	105-6548	High	36	190	3.5	A	A	A	A	A
2	105-7101	High	40	264	5.0	D	D	D	D	D
3	105-7391	High	34	314	4.6	A	A	A	NA	NA
4	105-7621	High	25	286	4.8	A	A	A	A	A/C
5	105-7717	High	35	269	5.2	A	A	A	A	A
6	105-7747	High	39	432	5.6	A	A	A	A	A
7	105-7895	High	36	91	4.2	A	A	A	A	A
8	105-7932	High	33	128	4.5	A	A	A	A	A
9	105-8136	High	36	139	4.2	D	A/D	A/D/A	D	A/D
10	105-8717	High	30	416	3.7	A	A	A	A	A
11	105-7726	High	48	92	5.3	A	A	A	A	A
12	105-7292	Low	59	187	5.7	A	A	A	A	A
13	105-7309	Low	27	173	4.9	A	A/C	A	A	A/C
14	105-7348	Low	34	228	4.8	A	A	A	A	A
15	105-7537	Low	35	387	4.4	A	A	A	A	A
16	105-7556	Low	44	52	5.1	A	A	A	A	A
17	105-7587	Low	31	366	4.7	D	A/D	A	A	A/D
18	105-7824	Low	34	223	4.9	A	A	A	A	A
19	105-7906	Low	32	89	4.8	A	A	A	A	A
20	105-8002	Low	53	239	4.0	A	A	A	A	A
21	105-8008	low	35	157	5.3	A	A	A	A	A
22	105-8073	Low	29	382	3.9	A	A	A	A	A
23	105-8132	Low	36	372	5.1	A	A	A	A	A
24	105-8198	Low	39	277	5.4	A	A	A	A	A
25	105-8221	Low	37	64	6.3	A	A	A	A	A
26	105-8222	Low	34	121	5.2	C	C	C	C	C
27	105-8422	Low	36	344	3.7	A	A	A	A	A
28	105-8441	Low	36	177	4.6	A	A	A	A	A
29	105-7492	Low	31	476	4.1	NS	A	A	A	A
30	105-8435	Low	NK	166	2.9	NA	NA	A	A	A

PBMC, peripheral blood mononuclear cell; NA, not amplified; NS, not sequenced; NK, not known; the PR and RT sequences did not have any drug resistance mutations; all sequences were R5 tropic based on genotypic predictions using V3 sequences.

TABLE 2. PRIMERS USED TO AMPLIFY THE *gag*, *pol*, AND *gp120* GENE PRODUCTS

Name	Genes	Position in HXB2	Sequence (5'→3')
691F	<i>gag</i>	691–714	GCAGGACTCGGCTTGCTGAAGCGC
734F		734–754	GCGGCGACTGGTGAGTACGCC
2033R		2015–2033	TTCCAACAGCCCTTTTTC
2046R		2027–2046	CTCCTTCCCACATTCCAAC
1828F	<i>pol</i>	1828–1846	ATGACAGCATGTCAGGGAG
2138F		2138–2158	AGAGCAGACCAGAGCCAACAG
3813R		3794–3813	AGGGAGGGGTATTGACAAAC
3887R		3865–3887	AGCTGCYCCATCTACATAGAAAG
HX5956F	<i>env</i>	5957–5986	TTAGGCATCTCCTATGGCAGGAAGAAGCGG
HX6556F		6557–6582	ATGGGATCAAAGCCTAAAGCCATGTG
HX7792R		7782–7811	AGTGCTTCCTGCTGCTCCCAAGAACCCAAG
HX7961R		7932–7961	TCTTGCTGGAGCTGCTTGATGCCCCAGAC

F stands for forward primers, R stands for reverse primers; nucleotide positions are based on the HXB2 reference strain.

Single molecule amplification of *gag* and *pol* gene products from 12 plasma samples was performed by using the Accu-Prime DNA polymerase and the methods previously described.^{4,11} Briefly, cDNA was diluted 4-fold and nested PCR was performed on each dilution. The highest dilution, yielding positive amplification, was selected and multiple reactions of the same dilution were tested for PCR amplification. When less than 40% of the reactions yielded positive PCR results, the dilution was considered to have single molecule amplification.⁷ All the amplified products then were sequence analyzed to determine if each variant sequenced was unique. A total of 39 *gag* and 28 *pol* variants were obtained and HIV-1 subtype and recombination analyses were performed on each variant. Seven plasma samples from subtype A, three recombinant subtypes, and one each of subtype C and D were included in single molecule analyses. All single molecule variant sequences clustered with population-based sequences obtained from the same sample, and the subtype and recombination patterns did not differ (data not shown).

Both strands of the DNA were sequenced by using overlapping primers and automated DNA sequencing. Sequences from individual primers were assembled using Sequencher Software, version 4.7 (Gene Codes Corporation). The assembled sequences and HIV-1 reference strains from the Los Alamos HIV database were aligned using the BioEdit sequence alignment and analysis software (www.mbio.ncsu.edu/bioedit/bioedit.html). After making alignments, the sequences were manually checked for quality and the open reading frames were determined. All the sequences were deposited in GenBank, accession numbers JQ364455–JQ364540, and KC513674–KC513730.

Phylogenetic trees were constructed for all the three genes (*gag*, *pol*, and *env*) separately (data not shown). HIV subtypes and intersubtype recombination were determined by using the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>) and REGA BIOAFRICA HIV-1 and HIV-2 subtyping and recombination tool version 2 (www.bioafrica.net/rega-genotype/html). This software uses phylogenetic methods to determine subtypes and bootscanning for recombination analysis. HIV-1 recombination was further analyzed by using the jumping profile hidden Markov model (jpHMM), which is freely available in the Los Alamos HIV database. jpHMM aligns a query sequence to a precalculated multiple sequence alignment of pure subtype sequences, predicts recombination

breakpoints, and assigns one of the given subtypes/genotypes to each segment of the sequence.¹³

When recombination was indicated, we constructed phylogenetic trees of the putative recombinant sequence by focusing on regions flanking the recombination breakpoints. The Molecular Evolutionary Genetics Analysis (MEGA) software version 4.1 (megasoftware.net/index.html) was used for phylogenetic analyses. Phylogenetic trees were constructed using the neighbor-joining method and Kimura's two-parameter correction with 500 bootstrap values. Sequences of pure subtypes/clades of the HIV-1 M group from the Los Alamos HIV database were included in the analyses.

HIV-1 coreceptor usage was predicted from the plasma V3 sequences by using WebPSSM and the 11/25 prediction methods.^{14,15} In the 11/25 prediction method, the presence of positively charged amino acids (K/R/H) at positions 11 and/or 25 predicts the presence of CXCR4 using viruses (X4). Conversely, the absence of positively charged amino acids at these positions predicts CCR5 using viruses (R5). HIV-1 genotypic drug resistance was determined by using the Stanford HIV drug resistance database (<http://hivdb.stanford.edu>). The Wilcoxon rank sum test was used to compare the CD4⁺ T cell count and viral load between the two exposure groups. We applied the Fisher's exact test to compare subtype distributions and the number of recombinant strains in the two groups.

In this group of HIV-infected Rwandan women, the overwhelming majority (80%) were infected with subtype A. We identified one patient with pure subtype C and another with pure subtype D infection. We identified four (13%) intersubtype recombinants, two each of A/C and A/D, out of the 30 samples analyzed (Table 1). There was no difference between the two exposure groups in HIV-1 subtype distributions or intersubtype recombination (Table 1). All sequences were predicted to use the CCR5 coreceptor (R5 tropic) and there were no major drug resistance mutations (data not shown).

To confirm that intersubtype recombination was correctly predicted, we performed detailed analyses of the sequences flanking the recombination breakpoints for all four patients who had recombinant viruses. The analyses for two are presented here and the analyses for the other two subjects will be made available upon request.

Plasma sequences from patient 105-7587 showed a complex recombination pattern (Fig. 1A–E). The sequences in the *gag*

FIG. 1. Detailed analyses of intersubtype recombination using phylogenetic and bootscan analyses of HIV-1 *pol*, *gag*, and *env* gene sequences obtained from study participant 105-7587. **(A)** HIV-1 *pol* sequences were analyzed for intersubtype recombination and an A/D recombination was predicted by using the jumping profile Hidden Markov Model (jpHMM), which is freely available in the Los Alamos HIV database. jpHMM is a probabilistic approach to compare a query sequence to a multiple alignment of multiple, single HIV-1 subtypes. **(B)** Phylogenetic analyses of the first 800 bp nucleotides of the *pol* gene. The HIV-1 sequences of patient 105-7587 in this portion of the gene clustered with subtype D in the phylogenetic tree. **(C)** Phylogenetic analysis of 688 bp nucleotides in the 3' *pol* gene of HIV-1 derived from patient 105-7587. Sequences from this portion of the gene clustered with subtype A in the phylogenetic tree. **(D)** Bootscan analysis of the *gag* gene: the *gag* gene was all subtype D with high bootstrap support. **(E)** Bootscan analysis of the gp120 gene: the *env*, gp120 was all subtype A with high bootstrap support.

region were of subtype D and it was confirmed by all three analyses methods. However, analyses of the *pol* sequences revealed recombination between subtypes A and D, and this was supported by bootscan analysis. The 5' portions of the *pol* sequences matched subtype D and the 3' region was subtype A (Fig. 1A). To confirm this observation, we constructed phylogenetic trees using sequences flanking the recombination breakpoints. First, we made two separate sequence alignments of the *pol* gene; the first part consisted of 800-bp sequences and the second portion had 690 bp. Representative sequences, from M group subtypes, in the Los Alamos HIV database were included in the analyses. Sequences of the first 800 bp clustered with subtype D reference strains in the

phylogenetic tree (Fig. 1B) and the 690-bp portions clustered with subtype A (Fig. 1C). The *gag* and gp120 region of this same patient were subtype D and A, respectively, by using bootscan analysis (Fig. 1D and E). The bootscan results were supported by phylogenetic tree analysis (Table 1).

Another subject, 105-7309, had recombinant sequences between subtypes A and C (A/C) in the *pol* region (data not shown). We constructed phylogenetic trees from sequences flanking the recombination breakpoint for this patient as well. First, we aligned 900-bp sequences from the 5' *pol* region with HIV-1 reference strains of the M group. In the phylogenetic tree, the sequences from this portion of the gene clustered with subtype A reference strains in the tree (data not shown).

Next, we analyzed 590-bp sequences from the 3' portion of *pol*. These sequences clustered with subtype C reference isolates in the phylogenetic tree (data not shown). This confirms that the *pol* gene is a recombinant between subtypes A and C. However, the *gag* and *gp120* sequences consistently clustered with subtype A, when analyzed with all three analytical methods (data not shown).

In this study, plasma and PBMC sequences were concordant in 28 out of 30 (93%) subjects. However, we identified two discordant HIV-1 sequences in plasma and PBMCs of two subjects, 105-7587 and 105-7621. Both subjects had inter-subtype recombinant viral strains involving subtype A, and non-A subtypes. The first subject, 105-7587, had an A subtype in PBMCs and D in plasma in the *gag* region, while the *pol* genes were an A/D recombinant in both plasma and PBMCs. This woman probably was dually infected with subtypes A and D. During the course of infection, the A/D recombinant strain might have emerged. It is also possible that she was dually infected with complex recombinant strains. However, it is difficult to determine with certainty what exactly took place.

The other subject, 105-7621, had an A/C recombination in the *pol* region in sequences obtained from the PBMC sample while the plasma sample was all subtype A in the *gag*, *pol*, and envelope gene. The *gag* region of the PBMC sequence was subtype A, similar to the plasma, and clustered with plasma sequences in the phylogenetic tree. This subject probably was dually infected or superinfected with a recombinant A/C and a pure subtype A virus. During the course of infection, the A subtype virus was probably more fit than the recombinant A/C variant and was actively replicating and was isolated in the plasma, while the A/C recombinant was found only in the archival PBMC samples. We cannot rule out the presence of dual pure subtype A and an A/C recombinant virus in PBMCs, as we did not perform detailed sequence analysis, including single molecule amplification and sequencing, as this was beyond the scope of this study. We also were not able to sequence HIV-1 *env* sequences from PBMC samples.

We studied women who were multiply raped during the 1994 Rwandan genocide. Some of the rapists told the women they were HIV-1 infected. We have also included women who were HIV infected, but did not report a history of rape and had only one lifetime partner. The results were not different in the two groups. We identified two of each inter-subtype recombinant in each of the two groups of women. One each of non-A, subtype C in the single partner group, and D in the multiply raped group were identified. The remaining 24 samples displayed subtype A in both plasma and PBMCs.

There may be multiple explanations for why the two groups of women displayed similar rates of intersubtype recombination. We do not know the date of infection for both groups of women, and it is possible that women in the multiply raped group may have been infected before or after the genocide. We also believe that since the samples were collected in 2005, 11 years after the Rwandan genocide, many of the women who had been multiply raped may have died before 2005 and therefore were not included in this study. It is possible that some of the women who were exposed to multiple infections with different or the same subtype were less likely to survive.^{16,17} Many of the men who were involved in raping may have been HIV uninfected. In many parts of sub-

Saharan Africa, most people did not know their HIV infection status in 1994. Moreover, HIV-1 prevalence in Rwanda has been relatively low; according to the Joint WHO/UNAIDS 2010 report, it stands at or around 3%. As reported in many studies, infection with multiple subtypes is not the rule. In the majority of primary infections only one variant establishes infection in the new host.¹⁸

There was no statistically significant difference in CD4 count and viral load in the two groups (Wilcoxon rank sum test). Moreover, there was no difference in subtype distribution and number of recombinant viruses between the two HIV-1 exposure groups (Fisher's exact test). The explanation for the similarity of the results between the two groups is unclear and may reflect various unknowns including HIV-1 exposure and cohort effects.

This study is the first to look into HIV-1 subtypes, recombination, drug resistance, and tropism in women who probably were exposed to HIV during the 1994 Rwandan genocide. Our report is based on studying plasma and PBMC samples and the analysis of all the three main HIV-1 genes, *gag*, *pol*, and *env*. Research on HIV-1 is disproportionately done on subtype B, which is prevalent in North America and Western Europe. However, the major epidemic is in sub-Saharan Africa and is caused by non-B subtypes. If subtype-specific interventions such as vaccines or small molecules targeting specific genes become available, it will be important to have current subtype information from countries affected by the epidemic.

Our study samples, both plasma and PBMCs, were from 2005 and are more representative of HIV-1 strains currently circulating in Rwanda. The data presented in this study show that recombination between HIV-1 variants is common and can readily be identified when large HIV-1 genomes are analyzed and multiple subtypes coexist. However, recombination is hard to detect within the same subtype.

We have previously reported intrasubtype recombination between viruses in the genital tract and blood of women infected with subtype B viruses.⁴⁻⁷ In those studies we had analyzed multiple variants by using limiting dilution PCR or cloning and analyzing multiple sequences. In this study, we first analyzed sequences obtained from population-based PCR amplifications from blood plasma samples. Only the predominant viral population is represented while minority variants are missed in this type of sequencing. To address this, we performed limiting dilution PCR and single variant analysis of 39 *gag* and 28 *pol* sequences on 12 plasma samples. However, the results were similar to the population-based sequence analysis. These results confirm that we did not miss any recombinant strains or dual infections. However, performing individual sequence analysis on all samples and all gene products was beyond the scope this study.

In summary, this study demonstrates that subtype A is still the prevalent subtype in Rwanda, accounting for 80% of all the subtypes in these Rwandan women. Moreover, despite lack of subtype diversity, HIV-1 recombinant strains were the second prevalent, 13%, in this study. This confirms that HIV-1 recombination is one of the driving forces of HIV diversity through generation of complex recombinant viruses. The prevalence of recombinant strains in this study was higher than what is found in the Los Alamos HIV database for Rwanda. This probably is due to the analyses of large HIV-1 gene fragments, including *gag*, *pol*, and *env*, as well as

studying both plasma and PBMC samples. This approach has increased the possibility of identifying complex recombinant viruses in this study.

Sequence Data

The GenBank accession numbers for the sequences described in this article are JQ364455–JQ364540 and KC513674–KC513730.

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Author Disclosure Statement

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