High Prevalence of Unique Recombinant Forms of HIV-1 in Ghana: Molecular Epidemiology From an Antiretroviral Resistance Study

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Background: In Ghana, programs to expand antiretroviral access are being implemented. In this context, the dynamic genetic evolution of HIV-1 requires continuous surveillance, particularly when diverse genetic forms co-circulate.

Methods: Phylogenetic and antiretroviral resistance analyses of HIV-1 partial *pol* sequences from plasma RNA samples from 207 Ghanaian individuals were performed.

Results: 66% of infections were CRF02_AG, whereas 25% were unique recombinant forms (URFs). All 52 URFs were characterized by bootscanning. CRF02_AG was parental strain in 87% of URFs, forming recombinants with genetic forms circulating in minor proportions: CRF06_cpx, sub-subtype A3, CRF09_cpx and subtypes G and D. Two triple recombinants (CRF02_AG/A3/CRF06_cpx and CRF02_AG/A3/CRF09_cpx) were identified. Antiretroviral resistance analyses revealed that six individuals, five of which were antiretroviral drug-experienced, harbored mutations conferring high level of resistance to reverse transcriptase inhibitors. No major resistance mutations were identified in the protease, although insertions of one and three amino acids were detected.

Conclusions: The high frequency of URFs detected probably reflects a significant incidence of coinfections or superinfections with diverse viral strains, which increases the genetic complexity of the HIV-1 epidemic in West Africa. Monitoring of HIV-1 drug resistance might provide data on the implications of intersubtype recombination in response to antiretrovirals.

Keywords: HIV-1, Ghana, subtype, recombinant, antiretroviral resistance

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INTRODUCTION

The origin of HIV-1 pandemic has been located in Central Africa.[1] Since then, viral strains of group M have evolved into a growing list of subtypes, sub-subtypes, and intersubtype recombinants.[2] The genetic diversity of HIV-1 strains circulating in the neighboring West African countries is being revealed with important implications on viral transmission, antiretroviral response, and vaccine design.[3–9] The cocirculation of different HIV-1 genetic forms, together with the frequently unknown HIV status of infected individuals, favors reinfections with genetically diverse viral strains. In this context, the extraordinarily high rate of viral genetic recombination facilitates the generation of intersubtype recombinants, which are becoming the main contribution to a rapid increase of HIV-1 genetic variability.[10] Those intersubtype recombinants that are identified in 3 or more epidemiologically unlinked individuals are designated as circulating recombinant forms (CRFs), and some of these are predominant in certain geographic areas.[11] Unique recombinant forms (URFs) are acquiring epidemiological relevance in the spread of HIV-1 in West Africa, as reported in Burkina Faso, Chad, Niger, Cameroon, Cote D'Ivoire, and Senegal[4–9] This dynamic evolution of HIV-1 in West Africa requires continuous surveillance as new genetic forms are being described.

Ghana is a West African country with 320,000 people estimated to be living with HIV and with a prevalence rate of 2.3% among adults.[12] Similar to its neighboring countries, where CRF02_AG is the dominant genetic form (between

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39% and 83%),[13] 63% of HIV-1 infections in Kumasi, the second largest city of Ghana, were reported to be caused by CRF02_AG.[14] This strain was found to recombine with other genetic forms in 20% of infections, whereas 5% were G/CRF06_cpx recombinants. After publication of this report, new genetic forms of HIV-1 have been described. Furthermore, antiretroviral therapy (ART) is expanding systematically with increased access at various health facilities throughout the country.[15,16] However, there is still inadequate information on antiretroviral susceptibility of the viral strains circulating in the country, as only a few studies provide descriptions on susceptibility of Ghanaian HIV-1 strains to ART.[17–20]

In this study, we analyzed the genetic characteristics of the protease (PR) and reverse transcriptase (RT) coding sequences of HIV-1 from infected patients, at the time when antiretroviral treatments were initiated in Ghana. As a result, we have obtained both a genotypic resistance profile and a detailed phylogenetic outline for an updated contribution to the molecular epidemiology of HIV-1 infections in Ghana.

MATERIALS AND METHODS

Patients and Samples

Blood samples were obtained from 207 Ghanaian HIV-1–seropositive persons accessing the national antiretroviral treatment program between 2002 and 2004 at 3 health facilities: St Martin's Hospital (Agormanya), Atua Government Hospital (both in the Eastern Region), and Korle Bu Teaching Hospital (in Accra, Greater Accra Region). These samples were collected as part of the routine clinical management of patients with HIV to inform initiation of ART. The HIV status of these patients was routinely ascertained by screening of blood samples for HIV antibody with rapid tests: Abbott Determine HIV-1/2 (Abbott Diagnostics, Abbott Park, IL) with confirmation of HIV type by INNO-LIA HIV I/II (Innogenetics, Gent, Belgium). The immune status of the patients was assessed by T $CD4^+$ cell counts with the FACSCount system (Becton Dickinson, Franklin Lakes, NJ), and plasma was stored at −70°C for further analyses.

RNA Isolation, Amplification, and Sequencing

Viral RNA was extracted from 1 mL of plasma with Nuclisens (BioMerieux, Marcy l'Etoile, France), in accordance with the manufacturer's protocol. An HIV-1 *pol* fragment (HXB2 positions 2080–3662) was amplified by reverse transcription, followed by nested polymerase chain reaction from plasma RNA using an in-house method.[21] Before sequencing, the polymerase chain reaction products were purified by digestion with exonuclease I and shrimp alkaline phosphatase (USB, Cleveland, OH). Direct sequencing in PR and RT coding regions was done with ABI Prism BigDye Terminator Cycle Sequencing kit and an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic Analysis

The sequences obtained were assembled with the SeqMan program (DNAStar, Madison, WI), and manually aligned using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Neighbor-joining trees, based on Kimura's 2-parameter distances, were generated using MEGA 3.1.[22] The reliability of tree topologies was assessed by bootstrapping with 1000 replicates, with a bootstrap support of 70%, or greater, being required to define a phylogenetic cluster. Recombination analyses were performed with bootscanning using Simplot v. 3.5.1.[23] In this analysis, windows of 250 nucleotides (nt) were used moving in 20 nt increments. Phylogenetic trees were constructed with the neighbor-joining method, using Kimura's 2-parameter distances, with the transition/transversion ratios estimated from the dataset using FindModel (http://hiv-web.lanl.gov/content/hiv-db/findmodel/findmodel.html).

Antiretroviral Resistance Analysis

The PR-RT sequences obtained were analyzed for antiretroviral resistance by the HIVdb program from Stanford University (http://hivdb6.stanford.edu/).[24] Mutation frequencies were compared at the HIV Mutation list from Stanford University (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivseq&action=showMutationForm).

Nucleotide Sequence Accession Numbers

Sequence data have been deposited in GenBank under accession numbers EU177879 to EU178085.

RESULTS

Phylogenetic Diversity of HIV-1 in Ghana

Phylogenetic analyses of the *pol* region of HIV-1 revealed the following subtype distribution of the 207 Ghanaian samples: 136 (65.7%) patients were infected with CRF02_AG, 8 (3.9%) with CRF06_cpx, 5 (2.4%) with subsubtype A3, 3 (1.4%) with subtype G, and 2 (1.0%) with CRF09_cpx and 1 patient was infected was with subtype B. Fifty-two (25.1%) samples were identified as unique recombinants: 17 (8.2%) were CRF02/CRF06, 16 (7.7%) CRF02/A3, 5 (2.4%) CRF02/CRF09, 3 (1.4%) CRF02/G, 2 (1.0%) CRF02/D, and one each of CRF06/CRF09, CRF06/A3, CRF09/A3, CRF09/G, CRF09/D, A3/D, and A3/G. Finally, 2 patients had HIV-1 strains, which were triple recombinants CRF02/A3/CRF06 and CRF02/A3/CRF09, respectively (Fig. 1). Neighbor-joining trees including only pure HIV-1 subtypes and CRFs are shown in Figure 2. Three patients, GH35, GH55 and GH220, had CRF02_AG/A3 recombinants with coincident breakpoint locations; however, unavailability of epidemiological data prevents us from proposing a putative new CRF. All other URFs had unique mosaic patterns in *pol*, as shown in Figure 3. A BLAST search was performed to carry out comparative phylogenetic analysis with sequences from Los Alamos HIV Sequence Database.[2] We did not find any similar recombination pattern in samples from the database.

CRF02_AG is the dominant genetic form of HIV-1 in Ghana, and it is parental of 87% of the URFs characterized in this study. Nevertheless, most of the other genetic forms, particularly A3 and CRF09_cpx, are found more frequently as part of URFs than as pure genetic forms (Fig. 1).

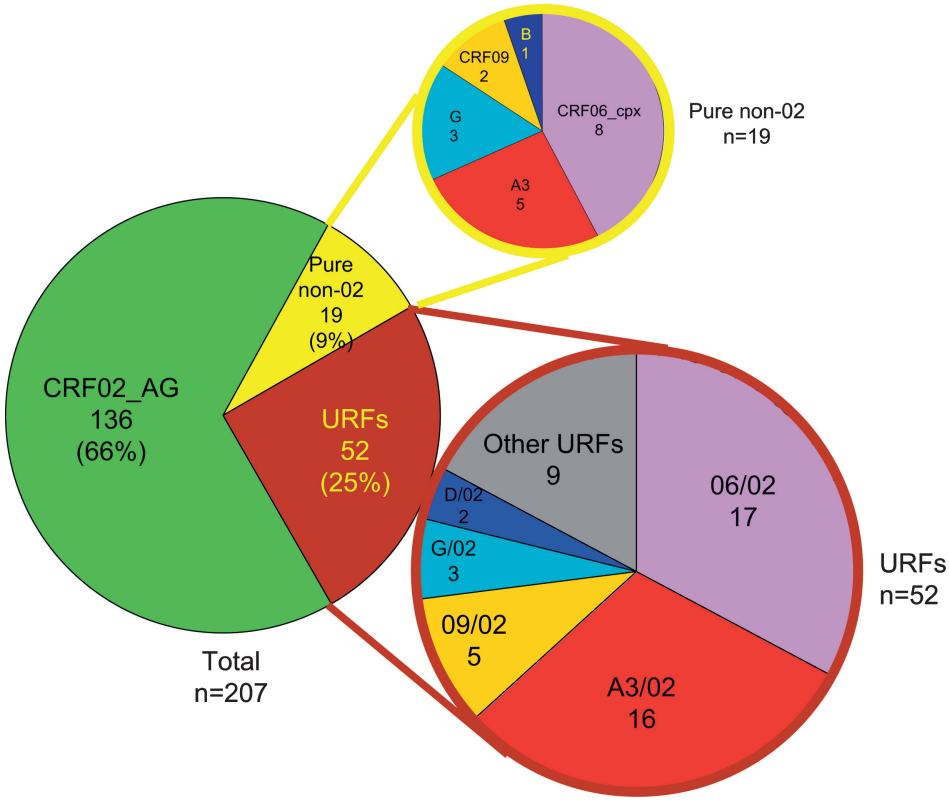

FIGURE 1. Distribution of HIV-1 genetic forms in Ghana, based on PR-RT sequences derived from 207 infected patients.

Reverse Transcriptase Drug Resistance Mutations

Reverse transcriptase drug resistance–associated mutations were detected in 20 patients. Six of them harbored mutations associated with high resistance level to RT inhibitors. Five of these patients had experienced ART before sampling for this study, and these high-resistance mutations correlated with their treatment regimens: the mutation M184V was present in 4 lamivudine-treated patients, T215Y was found in a zidovudine-treated patient, and 3 nevirapine-treated subjects had developed resistance mutations to nonnucleoside RT inhibitors (NNRTIs) (K103N and Y181C). The only HIV-1 subtype B sample identified in this study carried the mutation Y188L, which confers a high level of resistance to NNRTIs; however, in this patient, ART had been initiated after sampling for this study. Other mutations associated with intermediate, potential, or low-level resistance to nucleoside RT inhibitors (M41L, D67E, T69N/A, L210W, and K219T) or to NNRTIs (V108I, V179E/I, P225A, and K238T) were mostly detected among ART-naive subjects. Additionally, polymorphisms at resistance-related positions (T69S, K103R, and L210M) were found in 3 subjects. A complete list of the mutations associated with resistance to antiretroviral drugs that were found in the patients is given in Table 1.

Protease Drug Resistance Mutations

Although no major ART-resistance mutations were detected in the PR region, polymorphisms at resistance-associated positions were frequent, most of them subtype related. Mutations K20I and M36I were detected in 86% and 96% of the patients, respectively. Mutation V82I, which is common among subtype G strains, was found in 17 (8%) patients, including all subtype G samples. L10V, L10I, and V11I were detected in 25 (12%), 16 (8%), and 8 (4%) patients, respectively. Mutations A71T and L89V were detected in 1 patient each. More than 90% of the sequences had 3 polymorphisms in PR (I13V, M36I, and H69K) that are associated with resistance to tipranavir.[25]

Proteases from 2 patients (GH181 and GH162) had 1 insertion each of 1 (Ser) and 3 (Ala-Asn-Leu) amino acids after positions 37 and 35, respectively. Both samples were classified as CRF02_AG.

DISCUSSION

In this study, phylogenetic and antiretroviral resistance analyses were combined to determine the genetic profile of HIV-1 strains that circulated in Ghana at the start of the national ART program for HIV.

The analysis of the PR-RT regions of HIV-1 samples from 207 infected patients gave the following genetic classification: 66% were CRF02_AG, 25% URFs, and 9% minority "pure" genetic forms, including CRF06_cpx, sub-subtype A3, CRF09_cpx, and subtypes G and B. Three samples from different individuals shared the same mosaic pattern, comprising sub-subtype A3 and CRF02_AG, suggesting the existence of a new CRF. Each unique mosaic pattern was characterized by bootscanning. The diversity of URFs indicates the possible key role of superinfections or coinfections in this population. Also, it reflects that the genetic proximity of locally circulating genetic forms could favor recombination in the *pol* gene.

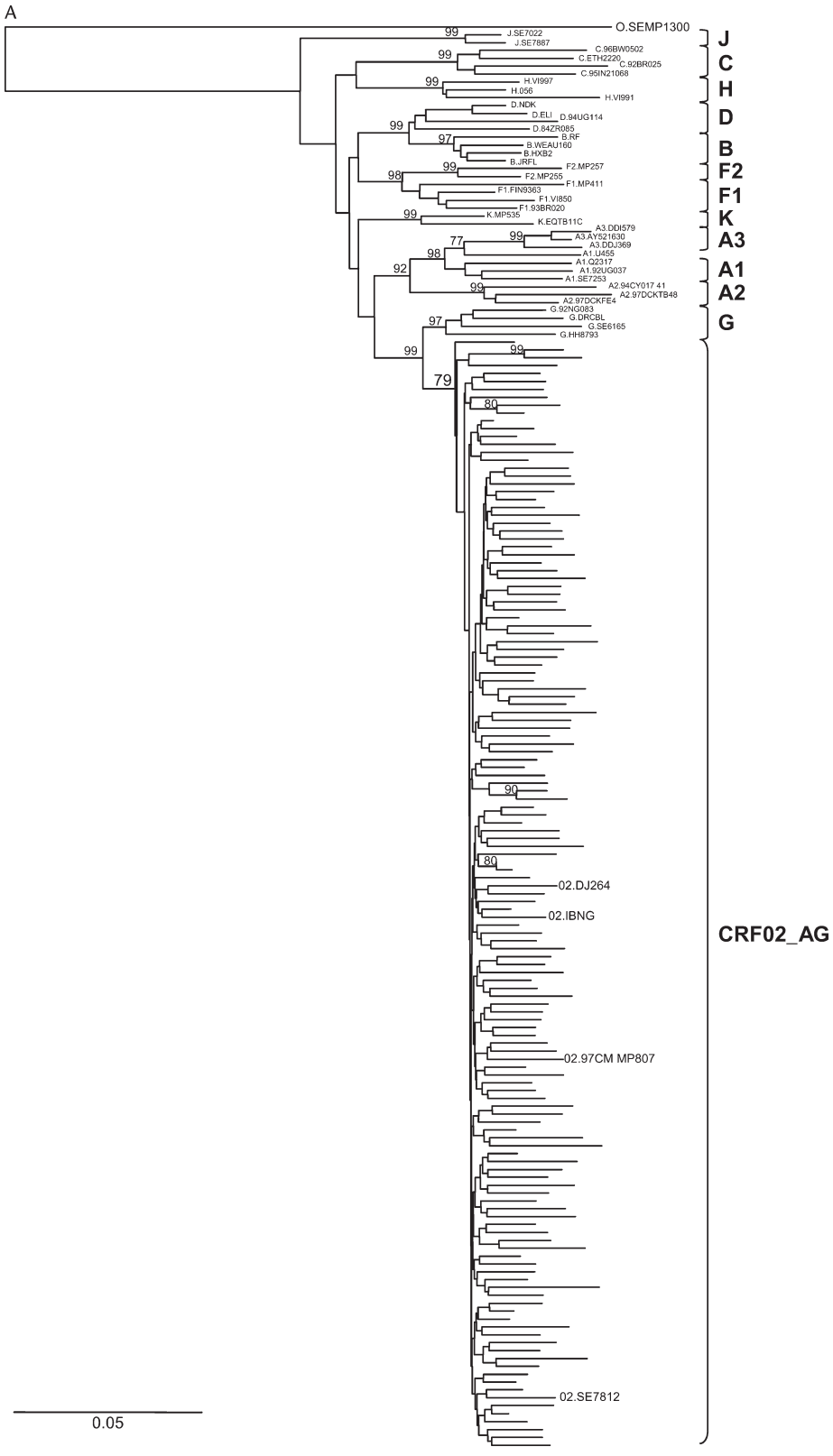

FIGURE 2. Neighbor-joining trees of 961 nucleotides in the PR-RT coding region of the HIV-1 pure subtype and CRF strains from Ghana, as determined by bootscanning with Simplot v.3.5.1. Bootstrap values ≥70% are shown. (A) One hundred thirty-six sequences classified as CRF02_AG in *pol*. Patient codes have been deleted for clarity. (B) Nineteen non-CRF02_AG pure genetic forms in *pol*. Sequences from the study are labeled with ''●''.

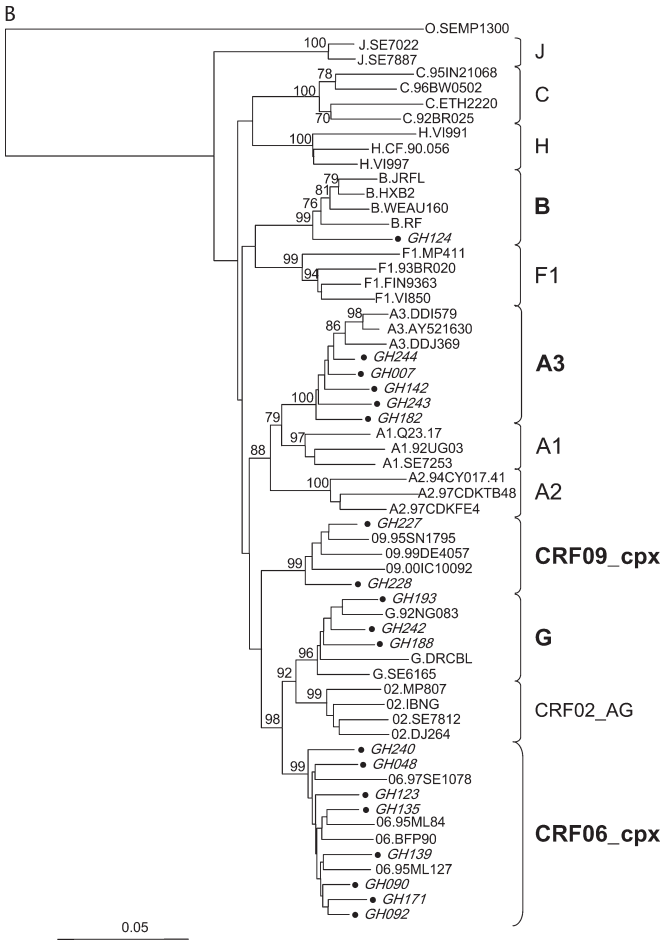

FIGURE 2. *(Continued).*

This distribution of HIV-1 genetic forms found in Ghana is concordant with reports derived from neighboring West African countries, where the proportion of URFs detected was between 9% and 27%.[5–7,26] However, in those studies, multiple regions of each genome were sequenced to identify recombination. In this study, CRF02_AG was found to be parental of 87% of the URFs characterized. Mosaics involving recombination of CRF02_AG with CRF06_cpx, CRF09_cpx, subtype D, subtype G, or sub-subtype A3 have also been identified in other West African countries.[4,8,9,26,27] However, this is the first report in which A3/G, A3/D, CRF09_cpx/CRF06_cpx, CRF09_cpx/G, and CRF09_cpx/D mosaics are described, including 2 triple recombinants. These data reveal the epidemiological relevance of second-generation recombinants in which different CRFs are involved.[7]

Multiple factors have to be considered to better understand this high prevalence of URFs. First, in West Africa, there is a wide cocirculation of different genetic forms, which may be due to the geographic proximity to the origin of initial expansion of HIV-1. Second, a low genetic distance between the cocirculating genetic forms could have facilitated recombination between homologous sequences.[28,29] In fact, the CRF02_AG and CRF06_cpx are related to subtypes A and G, and CRF09_cpx could share ancestors with CRF02_AG.[30] Third, these recombinants could have higher fitness than their parental genetic forms, as reported for CRF02_AG.[31,32] Fourth, infections with CRF02_AG strains are characterized by high viral loads, which facilitate virus transmission.[33] Fifth, these results could reflect a high frequency of multiple infections, in part due to transactional sex, that has been reported as the driving force in the dynamics of HIV-1 infection in Accra, Ghana.[34] The abundance of unique recombinants could also reflect a low cross-clade immunological protection between cocirculating genetic forms. In particular, the high degree of recombination of CRF02_AG reported in this study could be favored by a founder effect, where the subsequent introduction of the minority genetic forms frequently resulted in recombinants with CRF02_AG. In fact, it seems that some low-prevalence subtypes (F, G, H, J, and K) are spreading as part of recombinant strains more than as pure subtypes, possibly due to better fitness of the former.

It is possible that these observations could be an underestimation of actual recombination in HIV-1, as only a fragment of about 1100 nt in *pol* was analyzed by bootscanning. In fact, analyzing near full-length genomes of 7 samples, we found that sample GH142, which is non-recombinant A3 in *pol*, recombines with CRF02_AG in *env*.[35] Despite the underestimation, our results are comparable with those reported previously[14]: 63% (vs 66% in our report) CRF02_AG, 21% (vs 22%) recombinants containing CRF02_AG, and 16% (vs. 8%) other recombinants. In that report, 3 genes (*gag*, *pol*, and *env*) were partially sequenced, whereas in the present study, a longer fragment in *pol* has been sequenced, and the analysis has been improved by using bootscanning. Furthermore, CRF09_cpx and sub-subtype A3, which had not been characterized at the time of the previous publication, have now been included in the phylogenetic analyses. In fact, a recent report reveals that sub-subtype A3 has experienced a rapid rise in seroincident cases of HIV-1 in Senegal.[3]

Although full-length genome sequencing is the gold standard to characterize recombination, *pol* sequencing is useful for both screening for genetic diversity and analyzing antiretroviral susceptibility. In fact, analyzing the 31 CRFs with available mosaic patterns,[2] 27 (87%) of them show recombination breakpoints in *pol*. Therefore, analysis of recombination in *pol* is an accurate and cost-effective approach to conduct subtype classification, which will, in addition, provide information on antiretroviral susceptibility of HIV-1. The high recombination frequency in *pol*, also observed previously,[28,29] could play a key role in transmission of resistance mutations.

Most studies on HIV-1 antiretroviral (ARV) resistance in Africa have been performed in ARV- naive populations. In this study, most of the patients started ART after sampling, but 5 patients with high resistance level to RT inhibitors had ART previously. Nevirapine usage for prevention of mother to child transmission of HIV could be related to some of the NNRTI resistance mutations observed. We report 2 possible cases of transmission of drug resistance in ARV- naive patients (GH101 and GH124) who harbored viral strains with mutations

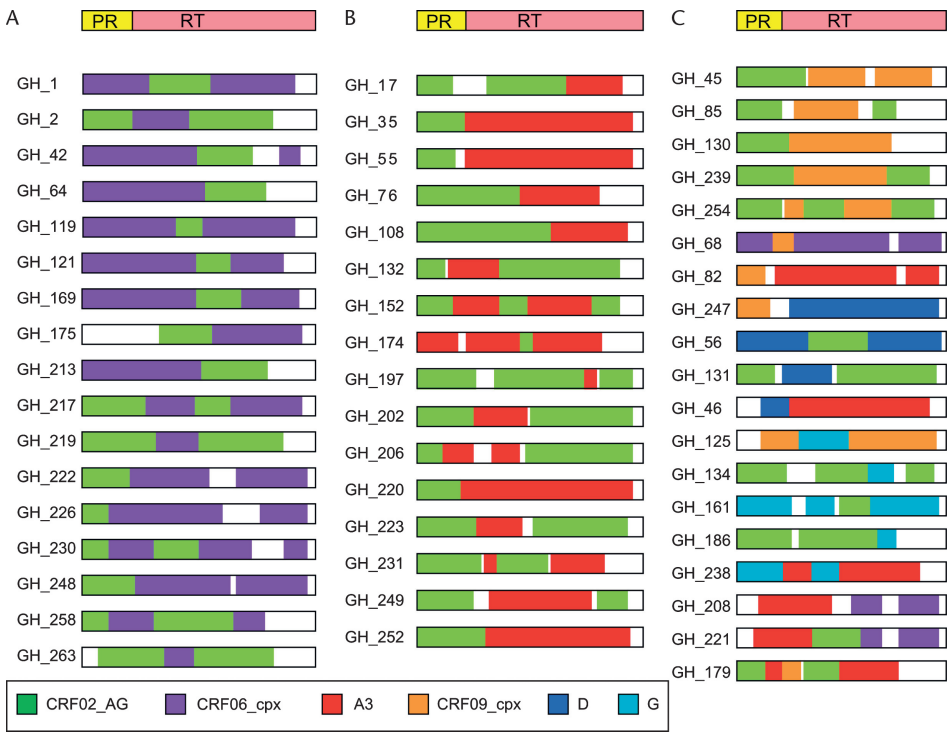

FIGURE 3. Mosaic patterns of the 52 URFs determined by bootscanning with Simplot v.3.5.1. Color bars represent segments clustering with reference sequences with bootstrap values ≥70%. (A) Seventeen CRF02_AG/CRF06_cpx recombinants. (B) Sixteen A3/CRF02_AG recombinants. (C) Other unique recombinants.

TABLE 1. Mutations/Polymorphisms At Antiretroviral Resistance-Associated Positions in HIV-1 Samples from Ghana

			Resistance-Associated Mutations†				
Sample	**Genetic Form**	**ART***	**PIs**	**NRTIs**		**NNRTIs**	**Resistance Level‡**
GH45	CRF02/CRF09	3TC		**M184V**			High
GH50	CRF02_AG	3TC, AZT		**M184V**	M41L		High
GH82	CRF09/A3	NVP (PMTCT)	L10I			**K103NK**	High
GH144	CRF02_AG	3TC, d4T, NVP		**M184V**		**Y181C**	High
GH212	CRF02_AG	3TC, AZT, NVP	L10I	**M184V**	D67E **T215YT**	**Y181C**	High
GH124	B	Naive	A71T			**Y188L**	High
GH101	CRF02_AG	Naive				P225A, K238T	Intermediate
GH64	CRF02/CRF06	NA			M41L		Low
GH135	CRF06_cpx	NA			M41L		Low
GH214	CRF02_AG	Naive			L210LW		Low
GH27	CRF02_AG	NVP (PMTCT)	L89V			V179E	Potential
GH38	CRF02_AG	Naive				V108IV	Potential
GH73	CRF02_AG	NA			T69N		Potential
GH80	CRF02_AG	NA			K219T		Potential
GH117	CRF02_AG	Naive				V179I	Potential
GH119	CRF02/CRF06	Naive	L10I			V179E	Potential
GH139	CRF06_cpx	Naive			T69AT	V108I	Potential
GH183	CRF02_AG	Naive			T69NT		Potential
GH215	CRF02_AG	Naive				V108IV	Potential
GH240	CRF06_cpx	Naive				V179E	Potential
GH123	CRF06_cpx	Naive				*K103R*	Susceptible
GH167	CRF02_AG	Naive			***L210M***		Susceptible
GH185	CRF02_AG	NA			T69S		Susceptible

*Antiretroviral treatment: AZT, zidovudine; 3TC, lamivudine; d4T, stavudine; NVP, nevirapine; PMTCT, prevention of mother to child transmission; NA: no data available.
†Mutations in bold type confer high resistance level according to HIVdb program from Stanford University. Polymorphisms at resistance-associated positions are given in italics.
‡Highest resistance level reported by HIVdb program from Stanford University.

conferring high or intermediate resistance level to NNRTIs. In patient GH124, the clinical records indicate no ART before sampling. A few days after blood was sampled, this patient started a triple ART (zidovudine, lamivudine, and efavirenz), and, subsequently, he experienced treatment failure, probably due to the presence of mutation Y188L. Afterward, the NNRTI was replaced by a ritonavir-boosted protease inhibitor (PI).

Different insertions in PR at a region between codons 35 and 38 have been described previously. Most of them are not related to a change in susceptibility to PIs, although they could contribute to a better fitness and present advantages for replication. The estimated frequency of HIV-1 viral strains with insertions in PR is 0.1%.[36] We suspect a subtype bias favoring this type of PR insertions, as the frequency in our study is about 10 times higher and both strains are CRF02_AG. This observation was corroborated by scrutiny of the Los Alamos HIV database, where 12 (0.8%) of the 1463 CRF02_AG genomes listed contained amino acid insertions in PR.[2]

The observed absence of PI resistance mutations is concordant with the data from other studies in Africa.[37,38] It also reflects the low usage of PIs in first-line therapy in Ghana. Subtype-related polymorphisms in the PR region, such as I13V, K20I, M36I, and H69K, could have an impact on PI susceptibility.[17,25,39] Long-term assessment of patients infected with non-B viruses receiving PIs will be needed to establish the clinical impact of these polymorphisms. In this regard, it has been reported that the HIV-1 PR from 39 drug- naive Ghanaian patients were differentially less susceptible to PIs than subtype B samples, suggesting implications in the response to antiretroviral treatments including PIs.[17]

This is the largest study on genetic susceptibility of Ghanaian HIV-1 strains to ARV drugs. The phylogenetic analysis of PR-RT coding sequences also reveals useful data on the molecular epidemiology of HIV-1 in Ghana. In this genome segment, the frequency of URFs is 25%, although, for a real estimation, complete genome sequencing is required. The high frequency of URFs found in this study suggests a significant role for reinfections with diverse viral strains in West Africa. Therefore, in locations where different genetic forms cocirculate, it is useful to analyze possible intragene recombinations by bootscanning or other methods. Surveillance of HIV-1 subtypes may have important implications for vaccine development efforts in West Africa.[40] These data highlight the need to promote more genetic studies on HIV in Africa, and both tasks, resistance and molecular epidemiology studies, could be simplified, compared with most published studies, by analyzing intragene recombination in PR-RT coding regions. Taken together these results, we can conclude that monitoring of HIV-1 drug resistance might provide data on the implications of intersubtype recombination in response to ART.[41]

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