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HIV Genetic Diversity in Cameroon: Possible Public Health Importance

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ABSTRACT

To monitor the evolving molecular epidemiology and genetic diversity of HIV in a country where many distinct strains cocirculate, we performed genetic analyses on sequences from 75 HIV-1-infected Cameroonians: 74 were group M and 1 was group O. Of the group M sequences, 74 were classified into the following env gp41 subtypes or recombinant forms: CRF02 ($n = 54$), CRF09 ($n = 2$), CRF13 ($n = 2$), A ($n = 5$), CRF11 ($n = 4$), CRF06 ($n = 1$), G ($n = 2$), F2 ($n = 2$), and E ($n = 1$, CRF01), and 1 was a JG recombinant. Comparison of phylogenies for 70 matched gp41 and protease sequences showed inconsistent classifications for 18 (26%) strains. Our data show that recombination is rampant in Cameroon with recombinant viruses continuing to recombine, adding to the complexity of circulating HIV strains. This expanding genetic diversity raises public health concerns for the ability of diagnostic assays to detect these unique HIV mosaic variants and for the development of broadly effective HIV vaccines.

HIV-1 strains from the west-central African country of Cameroon show remarkable genetic diversity. Cameroon is the only country where all three groups of HIV-1, M, N, and O cocirculate.[1–3] Almost all the known HIV subtypes and many recombinants, including circulating recombinant forms (CRFs) CRF01, CRF02, CRF04, CRF06, CRF09, CRF11, CRF13, and numerous unique recombinants, have been described.[4–8] Even recombination between HIV-1 group O and M strains has been documented in Cameroon.[9,10] It is well established that CRF02-AG and recombinants containing portions of the CRF02 genome are the most prevalent strains in Cameroon.[4–6,11] Continued genetic characterization of HIV-1 variants in this region, where multiple subtypes, CRFs, and unique recombinant forms (URFs) cocirculate, is important for global public health issues related to this diversity. For example, these complex recombinant viruses raise concern about whether any of these divergent strains might be missed by current diagnostic assays, as well as those assays currently under development.

Between September and November 1999, blood specimens were collected from 75 HIV-1-seropositive, antiretroviral drug-naive individuals, who routinely attended the tuberculosis (TB) and sexually transmitted infections (STIs) clinics in the seaport city of Douala and the capital city of Yaoundé, Cameroon. Blood specimens were screened for HIV using the rapid Abbott Determine HIV-1/2 assay (Abbott Laboratories, Abbott Park, IL) and two enzyme immunoassays, Genscreen HIV1/2 Version 2 (Pasteur Diagnostics, Paris, France) and AXSYM HIV1/2 gO (Abbott norge as, Oslo, Norway). Results from reactive specimens were confirmed by a Western blot test, HIV Blot 2.2 (Genelabs Diagnostics, Singapore).[12] Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by Ficoll-Hypaque gradient density, pelleted, and stored at −20°C. All individuals provided informed consent, although samples used in this study were unlinked to patient identifiers.

Viral DNA was extracted from PBMCs using the GFX TM Genomic blood DNA purification kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or the QIAamp DNA Mini Kit (QIAGEN Inc, Valencia, CA) according to the manufacturer's instructions. The env gp41 primary gp40F1-forward/gp41R1-reverse and nested gp46F2/gp48R2 polymerase chain reaction (PCR) primers, respectively, were used for amplification of a 445-bp fragment.[13] Nested PCR amplification of the entire protease (PR) gene with outside primers DP10-forward/DP11-reverse and inside primers DP16-forward/DP17-reverse was performed as pre-

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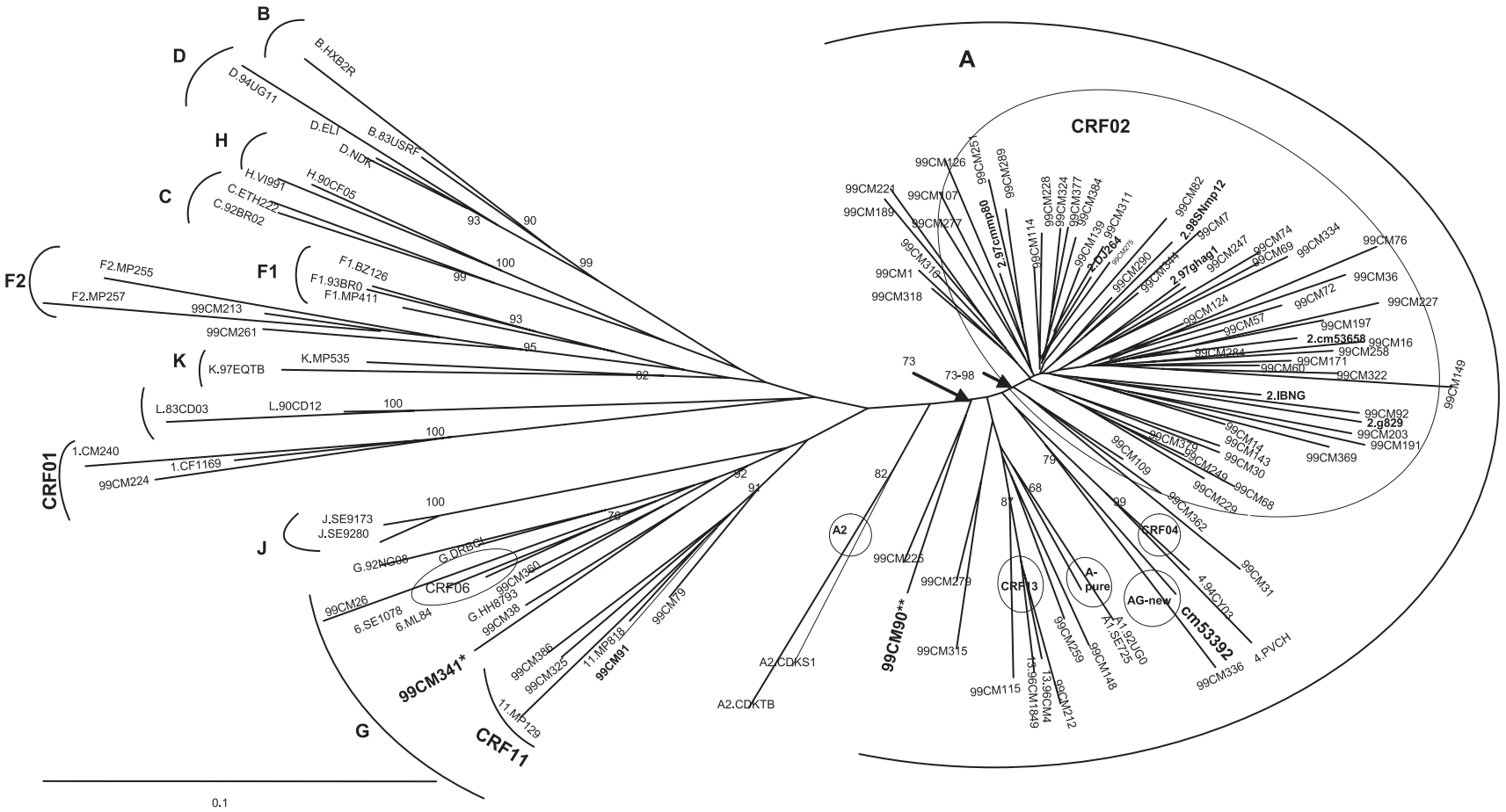

FIG. 1. Phylogenetic classification of 74 Cameroonian HIV-1, group M *env* gp41 sequences that are preceded by the prefix "99CM" indicating the year and the country (Cameroon) of specimen collection. An asterisk (*) marks the intersubtype G/J recombinant sequence and (**) indicates a sequence closely related to CRF09. HIV-1 references of group M are preceded by capital letters; CRFs are preceded by numbers; a new AG recombinant (cm53392) is in bold. The branches of subtype A are delineated. The numbers at the nodes correspond to bootstrap values; only values >70% are shown. The 73–98% for the CRF02 node indicates the range of bootstrap values for individual phylogenetic analyses of Cameroonian sequences. The scale bar indicates an evolutionary distance of 0.1; vertical distances are for clarity only.

Table 1. Sociodemographic and Genotypic Characteristics of HIV-1-Infected Patients from Cameroon

					HIV genetic subtype	
Sample ID	*Clinic*	*City*	*Age*	*Sex*	*gp41*	*PR*
99CM001	STI	Yde	25	M	CRF02_AG	CRF02_AG
99CM007	STI	Yde	28	F	CRF02_AG	CRF02_AG
99CM014	STI	Yde	26	F	CRF02_AG	CRF02_AG
99CM016	STI	Yde	28	F	CRF02_AG	CRF02_AG
99CM026	STI	Yde	24	F	CRF06_px	U
99CM030	TB	Yde	27	F	CRF02_AG	CRF02_AG
99CM031	TB	Yde	40	M	CRF02_AG	U
99CM036	STI	Yde	28	M	CRF02_AG	ND
99CM038	STI	Yde	28	M	G	G
99CM057	TB	Yde	40	F	CRF02_AG	CRF02_AG
99CM060	TB	Yde	35	M	CRF02_AG	CRF02_AG
99CM068	STI	Yde	40	M	CRF02_AG	CRF02_AG
99CM069	STI	Yde	27	M	CRF02_AG	CRF02_AG
99CM072	TB	Yde	45	M	CRF02_AG	F2
99CM074	STI	Yde	20	F	CRF02_AG	CRF02_AG
99CM076	STI	Yde	37	M	CRF02_AG	CRF02_AG
99CM079	STI	Yde	25	F	CRF11_cpx	CRF02_AG
99CM081	STI	Yde	27	M	O	O
99CM082	STI	Yde	52	F	CRF02_AG	CRF02_AG
99CM090	TB	Yde	40	M	CRF09_cpx	CRF02_AG
99CM091	TB	Yde	37	F	CRF11_cpx	CRF02_AG
99CM092	TB	Yde	62	F	CRF02_AG	CRF02_AG
99CM107	STI	Yde	26	F	CRF02_AG	CRF02_AG
99CM109	STI	Yde	24	F	CRF02_AG	CRF02_AG
99CM113	TB	Yde	37	F	CRF02_AG	CRF02_AG
99CM115	TB	Yde	38	M	CRF13_cpx	CRF02_AG
99CM124	TB	Yde	34	M	CRF02_AG	CRF02_AG
99CM126	STI	Yde	23	F	CRF02_AG	CRF02_AG
99CM139	STI	Yde	30	M	CRF02_AG	CRF02_AG
99CM143	STI	Yde	24	F	CRF02_AG	CRF02_AG
99CM148	STI	Yde	28	M	A	ND
99CM149	STI	Yde	30	F	CRF02_AG	CRF02_AG
99CM171	STI	Yde	39	F	CRF02_AG	ND
99CM189	STI	Yde	32	M	CRF02_AG	CRF02_AG
99CM191	STI	Yde	28	M	CRF02_AG	CRF02_AG
99CM197	STI	Yde	32	M	CRF02_AG	CRF02_AG
99CM203	STI	Yde	38	M	CRF02_AG	CRF02_AG
99CM212	STI	Yde	33	M	CRF13_cpx	CRF02_AG
99CM213	STI	Yde	33	M	F2	F2
99CM221	TB	Yde	NA	F	CRF02_AG	CRF02_AG
99CM224	STI	Yde	27	F	CRF01_AE	CRF01_AE
99CM225	STI	Yde	24	F	CRF09_cpx	CRF02_AG
99CM227	STI	Yde	39	M	CRF02_AG	CRF02_AG
99CM228	STI	Yde	60	M	CRF02_AG	CRF02_AG
99CM229	STI	Yde	44	F	CRF02_AG	CRF02_AG
99CM247	STI	Yde	27	M	CRF02_AG	CRF02_AG
99CM249	STI	Yde	29	F	CRF02_AG	CRF02_AG
99CM251	STI	Yde	22	F	CRF02_AG	CRF02_AG
99CM258	STI	Yde	30	F	CRF02_AG	CRF02_AG
99CM259	STI	Yde	53	F	A	CRF02_AG
99CM261	STI	Yde	21	M	F2	CRF11_cpx
99CM275	TB	Dla	35	M	CRF02_AG	CRF02_AG
99CM277	TB	Dla	48	F	CRF02_AG	CRF02_AG
99CM279	TB	Dla	29	M	A	CRF02_AG
99CM284	TB	Dla	38	M	CRF02_AG	CRF02_AG
99CM289	TB	Dla	36	F	CRF02_AG	CRF02_AG
99CM290	TB	Dla	31	M	CRF02_AG	U
99CM311	STI	Yde	30	M	CRF02_AG	CRF02_AG
99CM315	STI	Yde	39	F	A	CRF02_AG

TABLE 1. SOCIODEMOGRAPHIC AND GENOTYPIC CHARACTERISTICS OF HIV-1-INFECTED PATIENTS FROM CAMEROON (CONT'D)

					HIV genetic subtype	
Sample ID	*Clinic*	*City*	*Age*	*Sex*	*gp41*	*PR*
99CM316	STI	Yde	25	F	CRF02_AG	CRF02_AG
99CM318	TB	Yde	43	M	CRF02_AG	CRF02_AG
99CM322	STI	Yde	33	M	CRF02_AG	CRF02_AG
99CM324	STI	Yde	49	M	CRF02_AG	CRF02_AG
99CM325	STI	Yde	25	F	CRF11_cpx	CRF11_cpx
99CM334	STI	Yde	39	F	CRF02_AG	U
99CM336	STI	Yde	NA	M	$AG_{CM53392}$	$AG_{CM53392}$
99CM341	STI	Yde	20	F	G/J	CRF11_cpx
99CM344	STI	Yde	32	M	CRF02_AG	CRF02_AG
99CM360	TB	Dla	47	F	G	CRF02_AG
99CM362	TB	Dla	31	M	CRF02_AG	CRF02_AG
99CM369	TB	Dla	33	F	CRF02_AG	CRF02_AG
99CM377	TB	Dla	43	F	CRF02_AG	CRF02_AG
99CM379	TB	Dla	NA	NA	CRF02_AG	CRF02_AG
99CM384	TB	Dla	27	M	CRF02_AG	U
99CM386	TB	Dla	NA	NA	CRF11_cpx	ND

NA, not available; U, unclassified; ND, not done; Yde, Yaounde; Dla, Douala.

viously described.[14] The PCR amplification was performed with the Platinum Taq DNA Polymerase High Fidelity PCR system according to the manufacturer's instructions (Life Technology, Bethesda, MD). After purification (Qiagen, Valencia, CA), PCR products were directly sequenced using both forward and reverse nested PCR primers for HIV-1 gp41 and PR gene regions and resolved on an automated DNA sequencer ABI model 377 (Applied Biosystems, Foster City, CA). The derived nucleotide sequences were aligned using the Clustal W 1.83 multiple sequence alignment program included in the GeneStudio package (http://www.genestudio.com). Reference strains of groups M, N, and O were extracted from the Los Alamos databases (http://hiv-web.lanl.gov/MAP/hivmap.html). Phylogenetic analysis was performed by the neighbor-joining method, with the nucleotide distance calculated by Kimura's two-parameter method included in the Phylip package version 3.5c, with and without bootstrapping.[15] An HIV-2 Rod sequence was used as an outgroup. To avoid the influence of other sequences on the bootstrap value, confirmations of subtype assignments were performed separately for each sequence. The stability of the tree topology was tested by pruning, which consists of removing one sequence from an alignment and rerunning the phylogenetic analysis. For recombination analysis of DNA sequences, SimPlot software was used (SimPlot for Windows; http://sray.med.som.jhmi.edu/RaySoft/SimPlot); this program calculates and plots the percentage identity of the query sequence with reference sequences from group M viruses, in a sliding-window manner along the alignment with optimal step size.

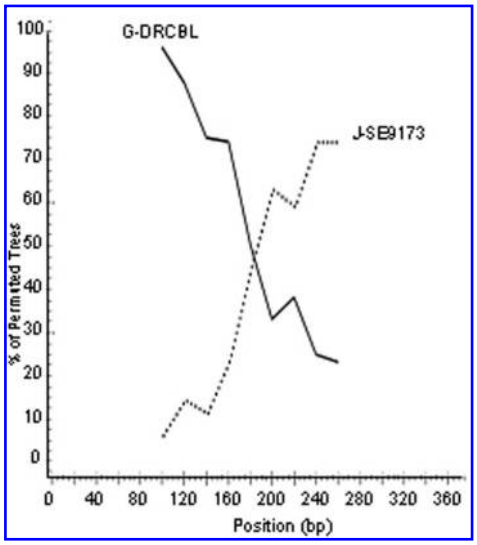

FIG. 2. Sliding window bootscan analysis (window 160 bp; step 30 bp) of the Cameroonian unclassifiable *env* gp41 sequence 99CM341 showing the recombination between subtypes G and J. The reference sequences were from the following subtypes: A (strain 92UG037), B (strain HXB2), C (strain ET2220), D (strain NDK), F1 (strain MP411), F2 (strain MP257), G (strain DRCBL), H (strain V19910, J (strain SE9173), K (strain EQTB11C), and potential L (strain 83CD03).

Phylogenetic analysis of 75 env gp41 sequences revealed that 74 clustered with HIV-1 group M (Fig. 1) while one clustered with group O (data not shown). The majority (85.0%, 63 of 74) of group M sequences fell within the subtype A clade**.** Fifty-four (85.7%) of the 63 subtype A sequences were further classified as CRF02, 2 as CRF13, 1 sequence clustered with a previously identified unique AG recombinant, CM_{53392}[8] (Fig. 1), and 2 sequences were found to be phylogenetically related to CRF09 (data not shown). Similarly, of seven sequences that clustered within the G clade five were further characterized: 4 grouped with CRF11-cpx reference sequences and 1 with CRF06-cpx. From the remaining 4 Cameroonian sequences, 2 were subsubtype F2, 1 was CRF01_AE, and 1 was unclassifiable (99CM341). Further pruning analysis revealed that the unclassifiable sequence clustered significantly (>80% bootstrap value) either with subtype G if subtype J was not included in phylogenetic analysis or with subtype J when subtype G sequences were absent (data not shown). These results suggested the presence of subtype G and J components in the

99CM341 gp41 sequence. Sliding-window bootscan analysis of this sequence showed clear intersubtype recombination with elements of subtypes G at the 5′ portion and J at the 3′ end (Fig. 2).

To estimate the extent of recombination involving CRFs circulating in Cameroon in 1999, we compared the *env* gp41 phylogenies for 70 HIV-1 group M and 1 group O sequences with the subtype/CRF designations of their PR genes (Table 1). There was concordance between the two gene regions for the group O strain, as well as for 74% (52 of 70) of the group M strains as follows: CRF02 (67.1%, $n = 47$), CRF11 (1.4%, $n = 1$), CRF01 (1.4%), cm53392 (1.4%), G (1.4%), and F2 (1.4%). The remaining 18 (26%) strains had discordant phylogenies with the following 10 gp41/PR mosaic structures: CRF02/unclass ($n = 4$), A/CRF02 ($n = 3$), CRF13/CRF02 ($n = 2$), CRF11/CRF02 ($n = 2$), CRF09/CRF02 ($n = 2$), CRF02/F2 ($n = 1$), G/CRF02 ($n = 1$), F2/CRF11 ($n = 1$), CRF06/unclass ($n = 1$), and intersubtype JG/CRF11 ($n = 1$). Overall, parallel analysis of the two gene regions, PR at 5′ and *env* gp41 at 3′-ends, revealed that only two (2.9%) viruses carried potentially "pure" subtypes (G and F2). Although CRFs were predominant (76%), almost 25% were unique recombinants with discordant subtype/CRF designations between PR and *env* gene regions. Consistent with CRF02 being the most predominant strain in Cameroon, most of the recombinant strains contained a fragment of CRF02.

Overall, our data demonstrated a broad diversity among HIV sequences from Cameroon, including gp41 and/or PR sequences from subtypes, CRFs, and URFs. Recombination is ongoing between these forms of HIV, adding to the increasing diversity in this region, and raising public health concerns about the ability of serologic and nucleic acid-based diagnostic assays to detect these complex variants of HIV. Especially since most nucleic acid assays used by blood banks for detecting recent, "window period" infections have not been thoroughly evaluated on these unique, complex forms of HIV. Studies are ongoing to evaluate Cameroonian serum or plasma samples, with at least partial subtype characterization, against a large battery of assays being used to diagnose HIV infections. The increasing genetic diversity of Cameroonian strains also presents a serious challenge for designing and developing broadly effective HIV-1 vaccines. Finally, the extensive recombination between circulating strains in Cameroon and other countries where multiple subtypes, CRFs, and URFs cocirculate continues to create a dilemma for classification of these complex forms of HIV.

SEQUENCE DATA

The sequences presented in this report have been deposited in GenBank under accession numbers DQ394108–DQ394177 for protease sequences and AY707001–AY707074 for gp41.

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