TABLE 1. Characteristics and Genetic Data for HIV-1 Infected Subjects Studied in Eastern Cameroon

Sample ID*	Age(y)	Sex	Nationality	Clinical Status	Genetic Subtype†			
					gag	*pol*	*env*-C2V3	gp41
01CM2213	25	M	CMR	ARC	CRF_01.AE	NA	CRF_01.AEA	NA
01CF2214	30	M	CAR	ARC	G	U	U	NA
01CM2215	34	F	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2216	36	M	CMR	ARC	A	NA	A	NA
01CM2217	26	M	CMR	ARC	CRF_11.cpx	NA	CRF_11.cpx	NA
01CM2218	25	F	CMR	AC	CRF_11.cpx	CRF_11.cpx	ND	U
01CM2219	32	F	CMR	ARC	CRF_11.cpx	NA	CRF_02.AG	NA
01CM2220	29	M	CMR	ARC	CRF_02.AG	NA	A	NA
01CM2222	46	M	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2223	25	M	CMR	ARC	CRF_01.AE	NA	CRF_02.AG	NA
01CM2224	29	F	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2225	31	M	CMR	ARC	B	NA	A	NA
01CM2226	32	M	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2227	30	F	CMR	AC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2228	42	F	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2229	30	F	CMR	AIDS	CRF_11.cpx	NA	CRF_11.cpx	NA
01CM2230	48	M	CMR	ARC	A	NA	A	NA
01CM2231	42	F	CMR	ARC	CRF_02.AG	NA	A	NA
01CM2232	19	F	CMR	AC	B	U	A	U
01CM2234	28	M	CMR	ARC	CRF_11.cpx	NA	CRF_02.AG	NA
01CM2235	33	M	CMR	ARC	B	U	ND	U
01CM2236	44	F	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2237	38	M	CMR	ARC	F2	NA	F2	NA
01CM2238	29	F	CMR	ARC	CRF_13.cpx	NA	CRF_01.AE	NA
01CM2239	25	M	CMR	ARC	CRF_13.cpx	NA	CRF_11.cpx	NA
01CF2240	50	M	CAR	ARC	CRF_02.AG	NA	CRF_13.cpx	NA
01CM2241	30	M	CMR	ARC	CRF_01.AE	CRF_11.cpx	ND	U
01CM2242	48	M	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2243	30	F	CMR	AC	CRF_11.cpx	CRF_11.cpx	ND	CRF_11.cpx
01CM2244	2	F	CMR	ARC	CRF_01.AE	NA	CRF_11.cpx	NA
01CM2246	17	F	CMR	ARC	B	NA	CRF_01.AE	NA
01CF2247	52	M	CAR	ARC	CRF_11.cpx	NA	CRF_01.AE	NA
01CM2248	28	F	CMR	ARC	CRF_01.AE	NA	A	NA
01CM2249	58	M	CMR	ARC	A	NA	A	NA
01CM2250	34	F	CMR	ARC	CRF_02.AG	CRF_02.AG	ND	U
01CM2252	34	M	CMR	ARC	CRF_02.AG	U	ND	U
01CM2253	21	F	CMR	ARC	CRF_01.AE	U	ND	A
01CM2256	35	M	CMR	ARC	CRF_01.AE	NA	A	NA
01CM2257	30	M	CMR	ARC	CRF_01.AE	NA	A	NA
01CM2260	29	F	CMR	ARC	CRF_13.cpx	U	A	CRF_13.cpx
01CM2262	27	F	CMR	ARC	B	NA	CRF_02.AG	NA
01CF2268	23	F	CAR	AC	CRF_02.AG	CRF_02.AG	ND	CRF_02.AG
01CM2269	21	F	CMR	ARC	CRF_11.cpx	CRF_11.cpx	ND	CRF_11.cpx
01CM2270	26	M	CMR	ARC	CRF_02.AG	CRF_02.AG	ND	U
01CM2271	26	F	CMR	ARC	CRF_11.cpx	CRF_02.AG	ND	CRF_11.cpx
01CM2272	27	F	CMR	ARC	CRF_11.cpx	NA	CRF_11.cpx	NA
01CM2273	54	M	CMR	ARC	CRF_11.cpx	NA	CRF_11.cpx	NA
01CM2274	35	F	CMR	ARC	CRF_02.AG	NA	CRF_02.AG	NA
01CM2275	35	M	CMR	ARC	CRF09_cpx	CRF_02.AG	ND	CRF09_cpx
01CM2276	30	F	CMR	ARC	CRF_11.cpx	NA	CRF_11.cpx	NA
01CM2277	28	F	CMR	ARC	CRF_11.cpx	CRF_11.cpx	ND	CRF_11.cpx
01CM2278	37	F	CMR	ARC	B	NA	CRF_02.AG	NA
01CM2280	26	M	CMR	AC	CRF_11.cpx	CRF_02.AG	ND	CRF_02.AG
01CM2281	20	F	CMR	ARC	CRF_02.AG	CRF_02.AG	ND	CRF_02.AG
01CM2284	25	M	CMR	AC	CRF_11.cpx	CRF_11.cpx	ND	CRF_11.cpx
01CF2287	33	F	CAR	AC	CRF_11.cpx	NA	CRF_01.AE	NA
02CM319	18	F	CMR	AC	ND	"O"‡	ND	"O"

*Subject identification number is preceded by year of sample collection; †genotyping of a part of *gag*-p24 (460 bp), *env*-C2V3 domain (approximately 550 bp), *pol*-In (288 bp), and *env*-gp41 (405 bp); ‡Group O from rural center province of Cameroon. AC, asymptomatic carrier; ARC, AIDS-related complex; CMR, Cameroon; NA, not available; ND, not detected.

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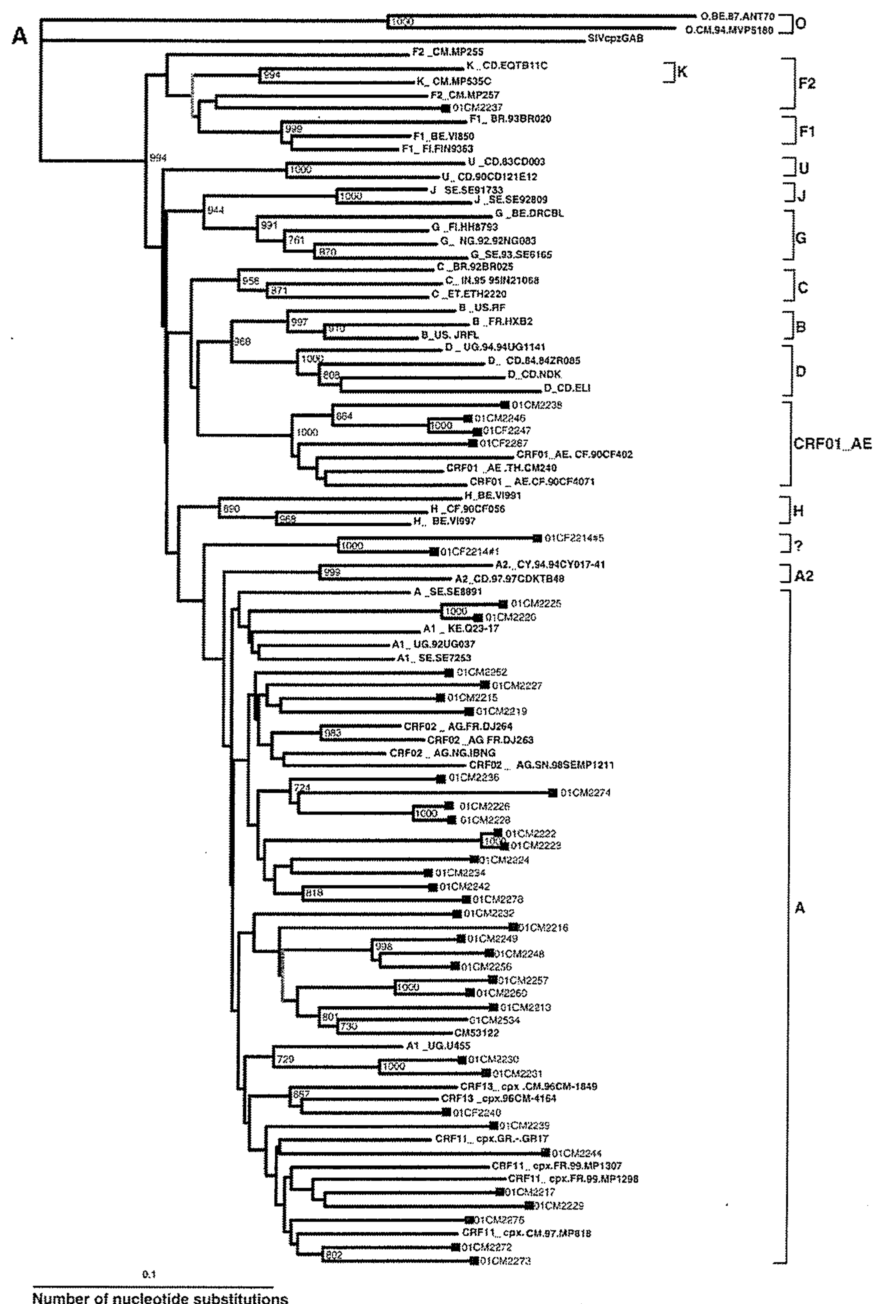

FIGURE 1. Phylogenetic trees based on a part of *env*-C2V3 gene (approximately 550 bp) of 40 HIV-1 strains (A), *gag* gene (approximately 460 bp) of 56 HIV-1 strains (B), *pol*-IN gene (288 bp) of 20 HIV-1 strains (C), and *env*-gp41 gene (approximately 405 bp) of 19 HIV-1 strains (D) from eastern Cameroon with reference sequences of representative subtypes/CRF. The reference sequences used in *gag, pol,* and *env* trees are shown in bold. The bootstrap value at each node represents the number among 1000 bootstrap replicates that support the branching order. Bootstrap value of ≥70% or higher is shown. Brackets on the right represent the major group M subtypes. Newly derived sequences are marked with a filled square (■). Further analysis of the 34 probable subtype A sample sequences without the rest of the sample sequences is shown in part E.

the 45 subtype A samples, 17 significantly clustered with CRF02_AG reference strains (with 74.6% bootstrap value).

Phylogenetic Analysis in the *env*-C2V3 and *gag*-p24 Genes

The summary of the phylogenetic analyses based on *gag*-p24 and *env*-C2V3 for the 57 HIV-1 strains is shown in Table 2. Out of the 40 samples that could be analyzed both in *gag* and *env*-C2V3 regions, 12 (30.0%) had discordant subtype or CRF designation: 2 subtype B/A (*gag/env*), 1 B/CRF01_AE, 2B/CRF02_AG, 1 CRF01_AE/CRF01_AE.A, 2 CRF11_cpx/CRF01_AE, 1 CRF13_cpx/A, 1 CRF13_cpx/CRF01_AE, 1 CRF13_cpx/CRF11_cpx, and 1 G/U. In 9 of these 12 strains, CRFs were involved in recombination events. Twenty-eight strains (70.0%) had the concordant subtypes or CRF designation between *gag* and *env:* 27 subtype A and 1 F2.

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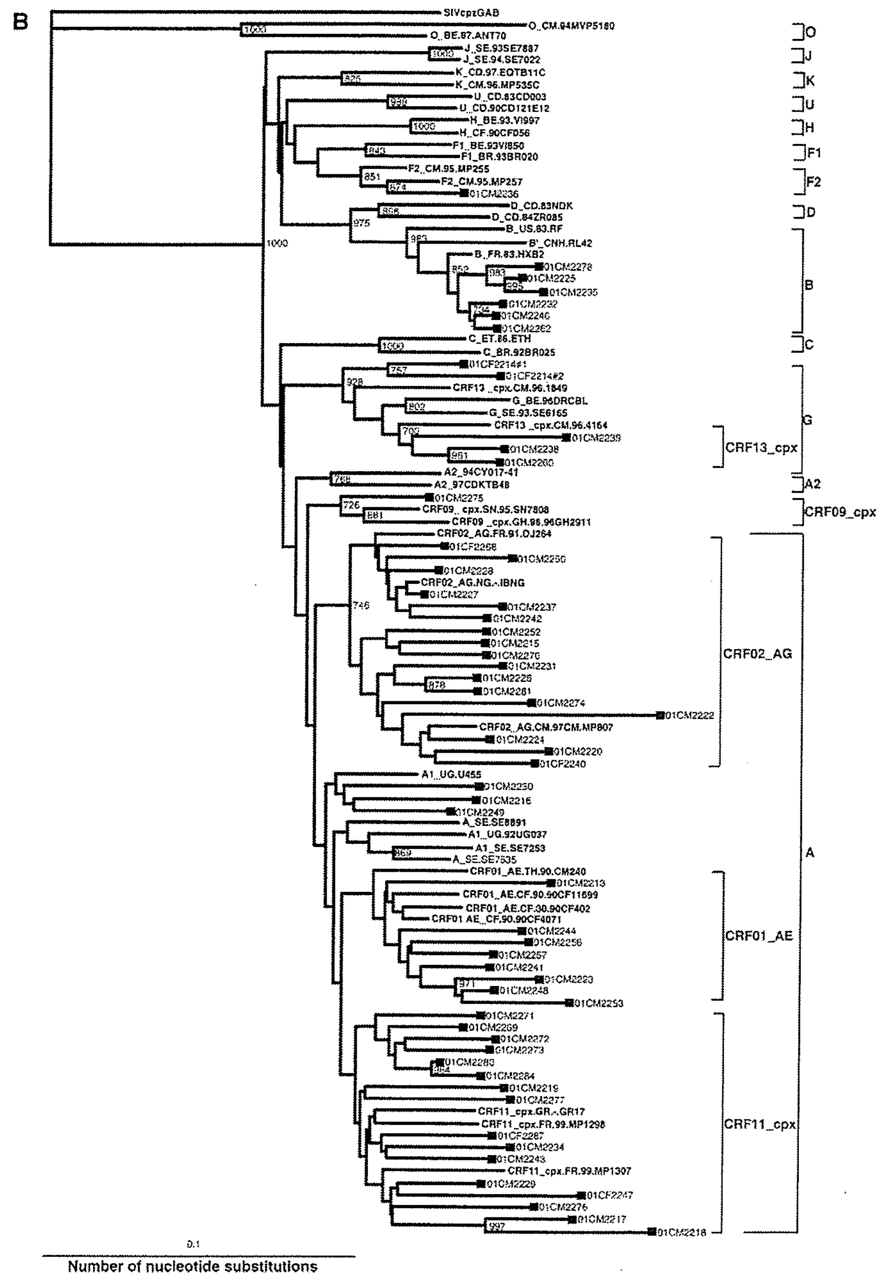

FIGURE 1. *(continued).*

Phylogenetic Analysis in the *pol*-IN and *env*-gp41 Genes

Additional PCR analyses on 17 samples with 2 *gag/env* profiles, (+/−) and (−/−), were carried out with groups M, N, and O primers for *pol*-IN and *env*-gp41. Phylogenetic analyses based on *pol*-IN and *env*-gp41 sequences revealed the presence of 10 profiles: 2 U/U, 1 U/A, 1 U/CRF11_cpx, 2 CRF11_cpx/U, 3 CRF11_cpx/CRF11_cpx, 1 CRF02_AG/CRF09_cpx, 1 CRF02_AG/CRF11_cpx, 2 CRF02_AG/U, 3 CRF02_AG/CRF02_AG, and 1 group O (*pol*-IN/*env*-gp41) (Fig. 1C and D). Thus, of the 17 HIV-1 strains negative for PCR with the *env*-C2V3 primers used, 10 (58.8%) had discordant subtype or CRF, and 7 (41.2%) had concordant one based on *gag/pol/env*-gp41 analyses (Tables 1, 2, and 3).

V3-Loop Amino Acid Sequence Analysis

The V3-loop has been shown to be important for antibody neutralization, phenotypic change, and co-receptor usage.[15] Figure 2 shows the predicted V3 amino acid sequence alignment of our 40 strains that could be analyzed in *env*-C2V3

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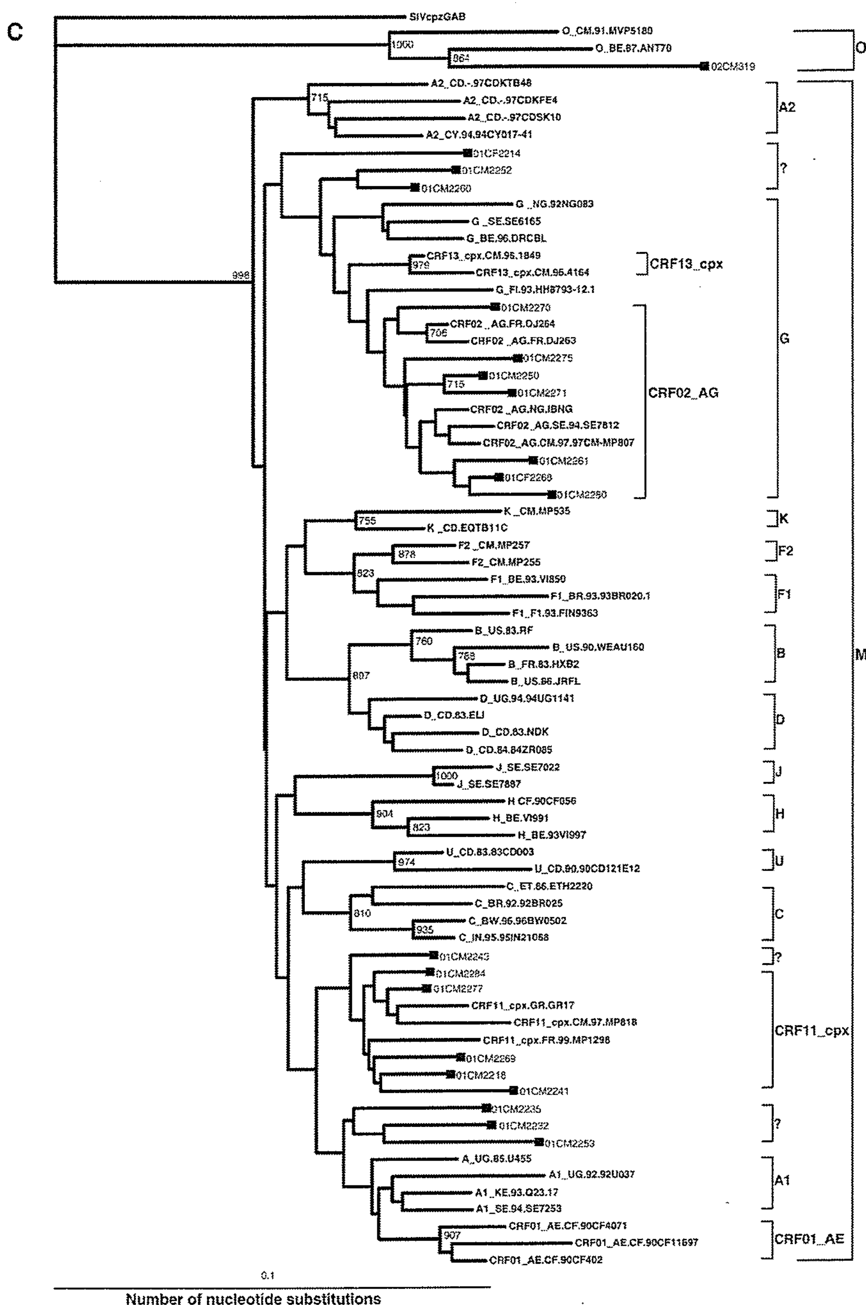

FIGURE 1. *(continued).*

region. Thirty-five, 3, and 2 of them had V3-loop consisting of 35, 36, and 37 amino acids in length, respectively. The most common tetrameric peptide at the apex of the V3-loop (highlighted gray in Fig. 2) was GPGQ (n = 34; 85.0%). A neutral amino acid residue, such as glycine or serine, was always present at position 11, and an amino acid with a negatively charged side chain, such as asparagic acid and glutamic acid, was present at position 25. The net charge of the V3-loop was from +2 to +6 (mean net charge 2.82; 95% CI 2.49–3.15). A common feature of the HIV-1 strains with V3-loop consisting of 36 and 37 amino acids was the insertion of an amino acid residue between positions 14–15 and 25–26.

DISCUSSION

In the current study we found that a high proportion (38.6%) of HIV-1 strains in eastern Cameroon were recombinants. This is higher than those reported in urban areas (7–10%) of Cameroon.[14,29] In our study, 4 subtype B/A (*gag/env*) recombinant strains were found in this area. This is the first report of intersubtype B/A, B/CRF01_AE, and B/CRF02_AG

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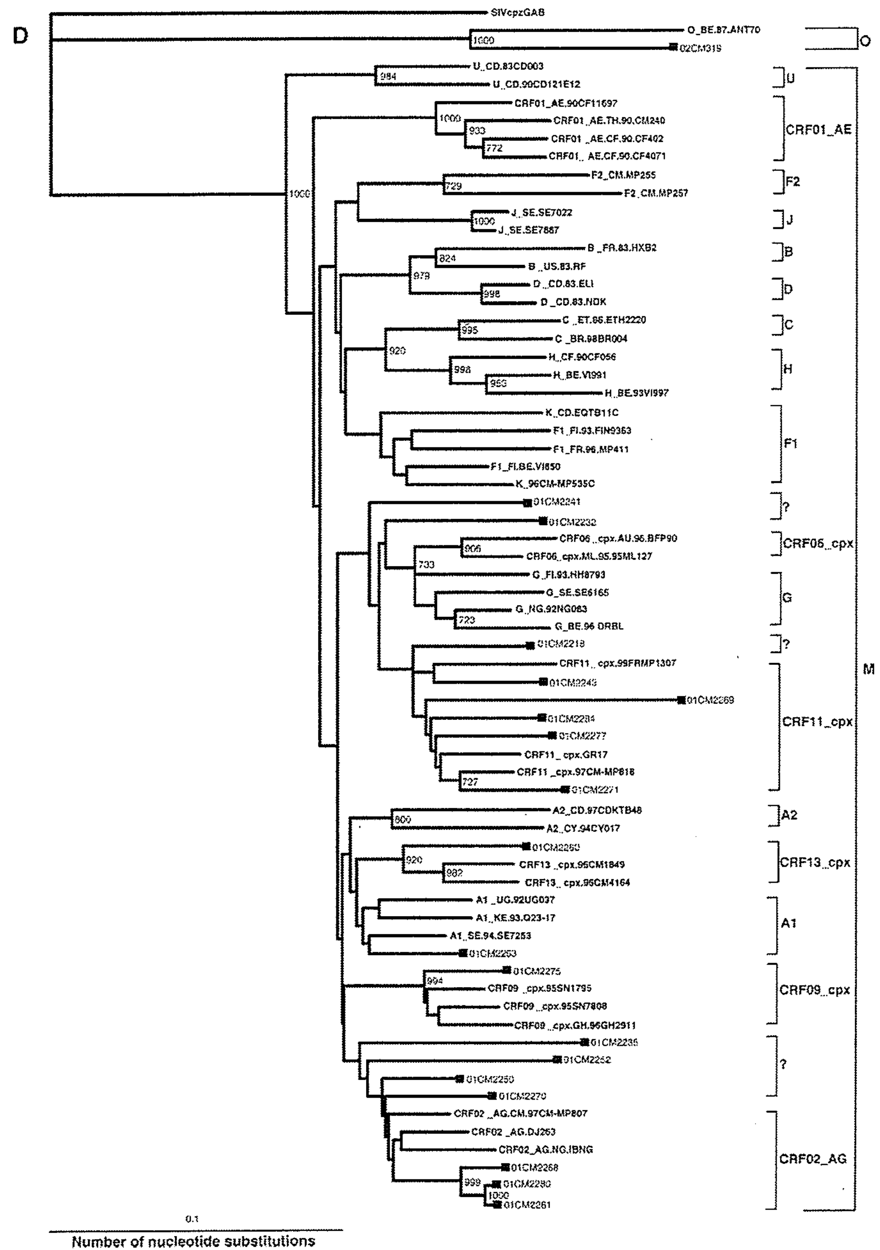

FIGURE 1. *(continued).*

in Cameroon. Although CRF15_01/B (*gag/env*) and CRF03_AB have been reported in Thailand and Russia, respectively, the mosaic composition of these CRFs is different from that of our B/A recombinants.[30,31] However, these 4 strains did not cluster together in *env*-C2V3 tree, suggesting that these viruses have evolved from independent recombination events. In addition, our strains 01CM2213 and 01CM2534[6] found in eastern and southern Cameroon, respectively, clustered significantly (with 80% bootstrap value) in *env*-C2V3 with a reference strain CM53122,[13] a recombinant between CRF01_AE and subtype A found in eastern Cameroon from an unlinked epidemiologic study, suggesting a possible emergence of a second generation of CRFs. These findings strongly suggest that this part of Cameroon is a potential hotspot of HIV-1 recombination, with a likelihood of an active generation of new forms of HIV-1 variants. It would, therefore, be important to monitor the appearance of new variants in this area.

Our data in rural eastern Cameroon highlight a relatively low prevalence of CRF02_AG (29.8 and 35.0%) and relatively high prevalence of CRF11_cpx (21.4 and 17.5%) based on *gag*-p24 and *env*-C2V3 analyses, respectively. These results are in contrast with previous reports on genetic diversity in

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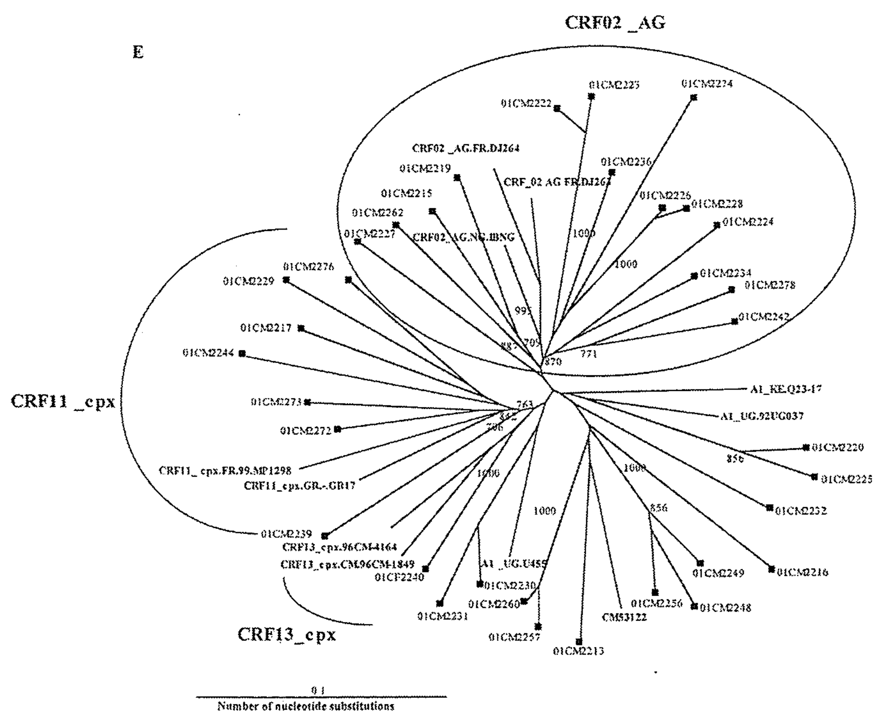

FIGURE 1. *(continued)*.

TABLE 2. Subtypes in *gag*-p24 and *env*-C2V3 for 57 HIV-1 Strains

	gag-p24	*env*-C2V3	n	Total
Concordant genotypes	A	A	3	
	F2	F2	1	
	CRF01 (A)	A	3	
	CRF01 (A)	CRF02 (A)	1	
	CRF01 (A)	CRF11 (A)	1	28
	CRF02 (A)	A	2	
	CRF02 (A)	CRF02 (A)	9	
	CRF02 (A)	CRF13 (A)	1	
	CRF11 (A)	CRF02 (A)	2	
	CRF11 (A)	CRF11 (A)	5	
Discordant genotypes	B	A	2	
	B	CRF01 (E)	1	
	B	CRF02 (A)	2	
	CRF01 (A)	CRF01 (A)	1	12
	CRF11 (A)	CRF01 (E)	2	
	CRF13 (G)	A	1	
	CRF13 (G)	CRF01 (E)	1	
	CRF13 (G)	CRF11 (A)	1	
	G	U	1	
Incomplete or negative PCR profile between *gag* and *env*	a*	ND	17	17

a*: 16 have (+/−) and 1 has (−/−) in the *gag/env* region.
ND, not detected.

Cameroon, where the majority of *env* and *gag* subtypes A viruses are CRF02-like.[7–15,18,29] The epidemiologic pattern of HIV-1 genetic diversity in eastern Cameroon, such as relatively low and high prevalence of CRF02_AG and CRF11_cpx, respectively, was more closely related to those of Chad and CAR than that of other geographic regions of Cameroon. A high proportion of subtype B (11%) was also found to be circulating in eastern Cameroon, and more interestingly, these subtype B sequences clustered significantly in the *gag*-p24 tree (with 80% of bootstrap value) with HXB2, a European subtype B reference strain. These findings could be related to the

TABLE 3. Subtypes in *pol* and *env*-gp41 for the 17 HIV-1 Strains With Incomplete and/or Negative PCR Profile Between *gag* and *env*-C2V3

	pol-Integrase (288 bp)				
env-gp41 (405 bp)	CRF02	CRF11	U	O	Total (%)
A			1		1 (5.9)
CRF02	3				3 (17.6)
CRF09	1				1 (5.9)
CRF11	1	3	1		5 (29.4)
U	2	2	2		6 (35.3)
O				1	1 (5.9)
Total (%)	7 (41.2)	5 (29.4)	4 (23.5)	1 (5.9)	17 (100)

U, unclassified; "O," group O.

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```
                      1   5     11   15   20   25    30   35       net              possible
Consensus-A           CTRPNNNTRKSIRI..GPGQAFYATGD.IIGDIRQAHC        charge     #aa  phenotype
01CM2215              ----G-KYK-KCTY..----DK-----.---IE----I         +3        35     R5
01CM2216              -I--Y-KYKQ-VR-.I-R--AV-A-S-KT-T-G-----         +5        37     R5
01CM2217              ------------HL..----------A.---N----N-         +3        35     R5
01CM2219              ---A--K-KGI.--..---RA---RDKVE-N---T---         +4        36     R5
01CM2220              ----------G-H-..----T-----A.-V------Y-         +3        35     R5
01CM2222              ---------R-VH-..----------N-.--------Y-        +2        35     R5
01CM2223              ---------R-V--..----------N-.--------Y-        +3        35     R5
01CM2224              ----GYKKERI-H-..---R-------.--T-------         +2        35     R5
01CM2225              -------A--G-H-..----S-F-AGE.----------         +2        35     R5
01CM2226              ---------T-VH-..----TFLCNR-.V-------F-         +2        35     R5
01CM2227              ---------R-IP-..----A---A--V-------Y--         +2        36     R5
01CM2228              ---------T-VH-..----T------.V-------F-         +2        35     R5
01CM2229              ----YH---R-V-LGI--RRV-----E.-R-N----Y-         +6        37     R5X4
01CM2230              -----------V--..----------E.---N------         +3        35     R5
01CM2231              -V---------V--..----------E.---N------         +3        35     R5
01CM2232              -V-------T--H-..---------G--.---------         +2        35     R5
01CM2234              VSDLG---------..-----------VT----K----         +2        36     R5
01CM2236              -----------VH-..----T-----A.------E---         +2        35     R5
01CM2239              ---TG-------H-..-----------.------K-E-         +2        35     R5
01CF2240              ---------Q-V--..----------E.---N------         +3        35     R5
01CM2242              ---------TG---..----------E--------Y--         +3        35     R5
01CM2244              -I--F-----G-HM..-----L----A.----P-K---         +2        35     R5
01CM2248              -I--G------V--..-----------.M-----K---         +3        35     R5
01CM2249              -V--G------V--..----T------.------K---         +3        35     R5
01CM2257              -V------------..---------R--.--------F-        +3        35     R5
01CM2260              -V---N---T--H-..---------R--.--------F-        +2        35     R5
01CM2262              -S--G----T-IH-..----A---G--.----------         +2        35     R5
01CM2272              -I--S-----G-H-..-----------.---N------         +2        35     R5
01CM2273              -I--------G-H-..----------.-.---S------        +2        35     R5
01CM2274              -I-------V-V--..----T-----A.-T-GFKPSI-         +3        35     R5
01CM2276              -I--G------VH-..---------DG.------E---         +2        35     R5
01CM2278              ---------R----..---RTFH---R.----------         +4        35     R5

Consensus-CRF01.AE    CTRPSNNTRTSITI..GPGQVFYRTGD.IIGDIRKAYC
CM53122               -V--N----R--H-..----A--AR--.-T----Q---
01CM2213              -V--ND------R-..-AR-TSLLCK-.-RRHN-GYKT         +5        35     R5
01CM2238              ----F-KI----FR-..---R--HA--G.-L-NT---S-        +5        35     R5
01CM2246              -----------VR-..--------G--E.----------        +2        35     R5
01CF2247              -----------VR-..--------G--E.----------        +2        35     R5
01CF2287              -----H-V----FR-..----------A.-T------F-        +2        35     R5
01CM2534              -I--N----QSIR-..----A--AR--.------Q---         +4        35     R5

Consensus-F1-F2       CTRPNNNTRKRIRIQRGPGRAFVTIGK..IGNMRQAHC
01CM2237              ----S-------H-..----T--AT-D.I--DI-K-Y-         +3        35     R5

HXB2                  CTRPNNNTRKRIRIQRGPGRAFVTIGK..IGNMRQAHC
01CF2214-1            ----------GV--..-----QTYATD.I--DI-----         +3        35     R5
Consensus-M           ----------S---..---Q---YATD.I--DI-----

                      ______________________________________
                                     V3_loop
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FIGURE 2. Alignment of deduced amino acid sequences of the V3-loop of HIV-1 strains from eastern Cameroon. Consensus amino acid sequences of subtype A, CRF01, subtype F2, and consensus M based on Los Alamos database are given on the top line using single letter code. Dashes and dots indicate identities and gaps, respectively. The V3-loop is indicated with a bar, and tetrameric tip of the V3-loop is highlighted in gray.

launch of a pipeline Chad–Cameroon project crossing the eastern part of Cameroon, in which not only local people but also >10,000 American and European engineers have been involved for >10 years. Further analysis of full-length genome sequences and appropriate phylogenetic analysis would be needed to clarify.

As for CRF09_cpx, which sequences are available only in Los Alamos database, has been found in independent studies in Senegal and Ghana, west Africa. This is the first report of CRF09_cpx among Cameroonian isolates in rural eastern Cameroon, suggesting that it may be spreading into west-central Africa.

In the current study we used 4 sets of primers to detect highly divergent HIV-1 strains. Among the 17 samples negative for PCR with the *env*-C2V3 primers used, 5 (29.4%) and 6 (35.3%) were unclassified in *pol*-IN and *env*-gp41 phylogenetic analyses, respectively. Despite the great genetic diversity and the high proportion (38.6%) of recombinant strains, all of the 17 samples could be detected with the *pol*-IN and *env*-gp41 primer sets. Thus, our PCR-based approach could be a molecular screening tool for detecting highly divergent HIV-1 variants.

To predict virtual phenotype of HIV-1, 2 approaches have been used based on subtype B analyses, such as the net amino acid charge and the presence of uncharged and negatively charged amino acids at positions 11 and 25 of the V3-loop.[32] In the current study, most of our strains have the V3-loop with less than +6 of net amino acid charge, suggesting that these HIV-1 strains were non-syncytium inducing viruses (NSI) using CCR5 as a coreceptor for cellular entry. In addition, of our 39 possible NSI viruses, 2 contained a positively charged amino acid residue at position 25 with a tetrameric motif consisting of GPGR at the apex of the V3-loop. The predicted phenotype did not correlate with the clinical status of our subjects, most of whom were AIDS-related complex or suspected AIDS patients. Recently, an independent study on non-B HIV-1 viruses has also revealed a lack of correlation between predicted phenotype of isolates based on V3-based genotype and in vitro syncytium-inducing

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capacity of the isolates from an urban area of Cameroon.[14] Further analysis will be needed for understanding of pathogenesis of non–subtype B HIV-1, and the relationship between V3-based genotype and phenotype of non–subtype B HIV-1.

In Cameroon, where multiple subtypes co-circulate, intersubtype recombination of HIV-1 strains is a common occurrence and plays a central role in active generation of new forms of HIV-1 variants. Monitoring the emergence of new strains not only in urban but also in rural areas would be very helpful for establishment of control strategies for the HIV epidemic in west-central Africa.

REFERENCES

1. Robertson DL, Anderson JP, Bradac JA, et al. HIV-1 nomenclature proposal. *Science*. 2000:288:55–56.
2. Gurtler LG, Hauser PH, Eberle J, et al. A new subtype of human immunodeficiency virus type 1 (MVP-5180) from Cameroon. *J Viral*. 1994;68: 1581–1585.
3. Simon F, Mauclere P, Roques P, et al. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nat Med*. 1998;4:1032–1037.
4. Corbet S, Muller-Trutwin MC, Vermisse P, et al. Env sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immuno-deficiency virus group N from the same geographical area. *J Virol*. 2000;74:529–534.
5. Zhong P, Burda S, Urbanski M, et al. HIV type 1 group M clades infecting subjects from rural villages in equatorial rain forests of Cameroon. *J Acquir Immune Defic Syndr*. 2002;31:495–505.
6. Ndembi N, Yumo H, Takehisa J, et al. HIV type 1 infection in pygmy hunter gatherers is from contact with Bantu rather than from nonhuman primates. *AIDS Res Hum Retroviruses*. 2003;19:441–445.
7. Heyndrickx L, Janssens W, Zekeng L, et al. Simplified strategy for detection of recombinant HIV-1 group M isolates by *gag/env* heteroduplex mobility assay. *J Virol*. 2000;74:363–370.
8. Janssens W, Buve A, Nkengasong JN, et al. The puzzle of HIV-l subtypes in Africa [editorial]. *AIDS*. 1997;11:705–712.
9. Nkengasong JN, Janssens W, Heyndrickx L, et al. Genotypic subtypes of HIV-1 in Cameroon. *AIDS*. 1994;8:1405–1412.
10. Takehisa J, Zekeng L, Ido E, et al. Various types of mixed infections in Cameroon. *Virology*. 1998;245:1–10.
11. Mboudjeka I, Zekeng L, Takehisa J, et al. HIV type 1 genetic variability in the northern part Cameroon. *AIDS Res Hum Retroviruses*. 1999;15:951–956.
12. Heyndrickx L, Janssens W, Ndumbe PM, et al. HIV-1 genetic diversity variability in Cameroon. *AIDS*. 2000;14:1862–1863.
13. Carr JK, Torimiro JN, Wolfe ND, et al. The AG recombinant IbNG and novel strains of group M HIV-1 are common in Cameroon. *Virology*. 2001;286:168–181.
14. Vergne L, Bourgeois A, Mpoudi-Ngole E, et al. Biological and genetic characteristics of HIV infections in Cameroon reveals dual group M and O infections and a correlation between SI-inducing phenotype of the predominant CRF02_AG variant and disease stage. *Virology*. 2003;310: 254–266.
15. Tebit DM, Zekeng L, Kaptue L, et al. Genotypic and phenotypic analysis of HIV type 1 isolates from western Cameroon. *AIDS Res Hum Retroviruses*. 2002;18:39–48.
16. Janssens W, Salminen MO, Laukkanen T, et al. Near full length genome analysis of HIV type 1 CRF02_AG subtype C and CRF02 _AG and subtype G recombinants. *AIDS Res Hum Retroviruses*. 2000;16:1183–1189.
17. Nyambi P, Heyndrickx L, Vereecken Katleen, et al. Predominance of infection with HIV-1 circulating recombinant form CRF02-AG in major Cameroonian cities and towns. *AIDS*. 2002;16:295–296.
18. Montavon C, Vergne L, Bourgeois A, et al. Identification of a new circulating recombinant form of HIV type 1, CRF11_cpx, involving subtypes A,G,J and CRF01_AE, in Central Africa. *AIDS Res Hum Retroviruses*. 2002;18:231–236.
19. Takehisa J, Zekeng L, Eiji I, et al. Human immunodeficiency virus type 1 intergroup (M/O) recombination in Cameroon. *J Viral*. 1999;73:6810–6820.
20. Peeters M, Liegeois F, Torimiro N, et al. Characterization of a highly replicative intergroup M/O human immunodeficiency virus type 1 recombinant isolated from Cameroonian patient. *J Viral*. 1999;73:7368–7375.
21. National AIDS Control Program. Yaounde, Cameroon: Ministry of Public Health; 2001.
22. Murphy E, Korber B, Georges-Courbot MC, et al. Diversity of V3 region sequences of human immunodeficiency viruses type 1 from Central African Republic. *AIDS Res Hum Retroviruses*. 1993;10:997–1006.
23. Muller-Trutwin MC, Chaix ML, Letourneur F, et al. Increase of HIV-1 subtype A in Central African Republic. *J Acquir Immune Defic Syndr*. 1999;21:164–171.
24. Vidal N, Koyalta D, Richard V, et al. High genetic diversity of HIV-1 strains in Chad, west Central Africa. *J Acquir Immune Defic Syndr*. 2003;33:239–246.
25. Peeters M, Toure-Kane C and Nkengasong JN. Genetic diversity of HIV in Africa: impact on diagnosis, treatment, vaccine development and trials. *AIDS*. 2003;17:2547–2560.
26. Yang C, Pieniazek D, Owen SM, Fridlung C, et al. Detection of phylogenetically diverse human immunodeficiency virus type 1 groups M and O from plasma by using highly sensitive and specific generic primers. *J Clin Microbiol*. 1999;37:2581–2586.
27. Kimura M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotides sequences. *J Mol Evol*. 1980;16:111–120.
28. Saitou N and Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Bio Evol*. 1987;4:406–425.
29. Montavon C, Toure-Kane C, Liegeois, et al. Most *env* and *gag* subtype HIV-1 viruses circulating in west and west central Africa are similar to the prototype AG recombinant virus IBNG. *J Acquir Immune Defic Syndr*. 2000;23:363–374.
30. Tovanabutra S, Watanaveeradej V, Viputtikul K, et al. A new circulating recombinant form, CRF15_01B, reinforces the linkage between IDU and heterosexual epidemics in Thailand. *AIDS Res Hum Retroviruses*. 2003;19:561–567.
31. Liitsola K, Holm K, Bobkov A, et al. An AB recombinant and its parental HIV type 1 strains in the area of the former Soviet Union: low requirements for sequence identity in recombination. *AIDS Res Hum Retroviruses*. 2000:16:1047–1053.
32. Jensen MA, Van't Wout AB. Predicting HIV-1 coreceptor usage with sequence analysis. *AIDS Rev*. 2003;5:104–112.

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AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 20, Number 12, 2004, pp. 1352–1357
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Sequence Note

Genetic Diversity of HIV Type 1 in Likasi, Southeast of the Democratic Republic of Congo

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ABSTRACT

To investigate the prevalence of subtypes A and C, and the existence of recombinants of both subtypes in the southeast of the Democratic Republic of Congo (DRC), blood samples were collected from 27 HIV-infected individuals in Likasi, located in an area bordering close to Zambia, and analyzed phylogenetically. Out of the 24 strains with a positive PCR profile for *pol*-IN and *env*-C2V3, 15 (62.5%) had a discordant subtype or CRF designation: one subtype A/G (*pol/env*), four A/U (unclassified), three G/A, one G/CRF01, three H/A, one J/C, one CRF02 (G)/A, and one U/A. Nine (37.5%) strains had a concordant subtype or CRF designation: five subtype A, two C, one D, and one CRF02/G. The remaining three samples negative for PCR with *env*-C2V3 primers used in this study were further analyzed with *env*-gp41 primers and revealed the presence of two profiles: two J/J (*pol*-IN/*env*-gp41) and one C/G. These data highlight the presence of a high proportion (16/27, 59.3%) of recombinant strains and a low prevalence (4.1 and 7.4%) of subtype C based on *env*-C2V3 and *pol*-IN analyses, respectively, in Likasi. In addition, this is the first report that CRF02_AG exists in DRC, though the epidemiological significance of the existence of CRF02_AG in DRC remains unknown.

HUMAN IMMUNODEFICIENCY VIRUS TYPE-1 (HIV-1) has been classified into three major phylogenetic groups, termed M (major), N (non-M, non-O), and O (outlier).[1] The vast majority of variants found worldwide and responsible for the AIDS pandemic belong to group M.[2,3] Phylogenetic analysis of group M has further led to its subclassification into nine pure subtypes, A–D, F–H, J, and K and subsubtypes A1, A2, F1, and F2.[2] Recently, it was realized that a significant fraction of HIV-1 isolates, 10–40% or more, exhibit a shift in subtypes when different regions of their genome are analyzed.[3] Currently, some of the mosaic HIV-1 genomes play a major role in the global AIDS epidemics and are designated as circulating recombinant forms (CRFs), CRF01-CRF16. Although subtypes A, C, and CRF02_AG are most prevalent in Africa, the distribution of CRF/subtype is very heterogeneous.[2–7] The proportion of CRF02_AG among subtype A strains based on *env* sequences decreases from west to central Africa, with an absence of CRF02_AG in the Democratic Republic of Congo (DRC).[2,8,9] The profile of HIV-1 endemic in DRC, such as high number of cocirculating HIV-1 subtypes, possible recombinant viruses, and unclassified strains, is consistent with that of an old and mature epidemic of HIV-1.[5]

The DRC is bordered on the southeast by Zambia. The majority (95%) of the HIV-1 strains circulating in Zambia are subtype C, although HIV-1 group M subtypes A, D, G, and J as well as group O have been identified.[2,3,10,11]

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The purpose of this study was to investigate the prevalence of subtypes A and C, and the existence of the recombinants of both subtypes in Likasi, located in the southeast of the DRC, 200 km from Lubumbashi in an area bordering Zambia.

Blood samples (10 ml) were collected from 27 HIV-1 infected individuals in February and September 2001. Plasma and buffycoat were separated and stored at −80°C until use. The plasma samples were screened for HIV antibodies with a commercial particle agglutination (PA) test kit (Serodia-HIV, Fujirebio, Tokyo, Japan).

Genomic DNA was extracted from buffycoat of the seroreactive samples using a Qiagen DNA extraction kit (Qiagen, Hilden, Germany). A part of the *pol* gene coding integrase (IN) (corresponding to 4493–4780 nt in HIV-1_{HXB2}) and *env* gene covering C2V3 (corresponding to 6975–7520 nt in HIV-1_{HXB2}) was amplified with nested polymerase chain reaction (PCR) using the primers unipol 5 (5′-TGGGTACCAGCACACAAAGGAATAGGAGGAAA-3′)/unipol 6 (5′-CCACAGCTGATCTCTGGCCTTCTCTGTAATAGACC-3′) and M5 (5′-CCAATTCCCATACATTATTGTGCCCCAGCTGG-3′)/M10 (5′-CCAATTGTCCCTCATATCTCCTCCTCCAGG-3′), respectively, in the first round, and unipol 1 (5′-AGTGGATTCATAGAAAGCAGAAGT-3′)/unipol 2 (5′-CCCCTATTCCTTCCCCTTCTTTTAAAA-3′), and M3 (5′-GTCAGCACAGTACAATGACACATGG-3′)/M8 (5′-TCCTTGGATGGGAGGGGCATACATTGC-3′), respectively, in the second round.[11] Nested PCR was performed with an AmpliTaq Gold PCR kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Amplification was done with one cycle at 95°C for 10 min, and 35 cycles at 95°C for 30 sec, 45°C for 30 sec (for the *pol* region) or 55°C for 30 sec (for the *env* region), and 72°C for 1 min, with a final extension of 72°C for 10 min. Samples that could not be amplified with the *env*-C2V3 primers were analyzed with *env*-gp41-specific primers.[12] PCR amplification was confirmed by visualization with ethidium bromide staining of the gel. The PCR products were cloned by using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) and plasmid DNA for sequencing was prepared by a plasmid miniprep kit (Sigma, Hilden, Germany). DNA sequencing was carried out using dye-deoxy terminator chemistry on an ABI 310 automatic sequencer (Applied Biosystems, Foster City, CA). We sequenced at least 12 clones to obtain a consensus sequence. Sample DNA sequences were aligned with subtype reference sequences from the Los Alamos database by CLUSTAL W (version 1.81) with subsequent inspection and manual modification. The frequency of nucleotide substitution in each base of the sequences was estimated by the Kimura two-parameter method.[13] Phylogenetic trees were constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications.[14] All alignments were gap stripped for the generation of trees. The profile of the tree was visualized by Treeview version 1.6.5.

The phylogenetic trees based on *env*-C2V3 and *pol*-integrase sequences were constructed with representative HIV-1 strains of each subtype and CRF as a reference (Fig. 1). Out of the 27 samples from Likasi, 24 (88.9%) were found to be positive for HIV-1 PCR with *pol*-IN and *env*-C2V3 primers. The remaining 3 (11.1%) samples were negative for PCR with the *env*-C2V3 primers used in this study. The phylogenetic tree based on *env*-C2V3 sequences (Fig. 1A) showed that out of the 24 samples 13 were subtype A, three C, one D, two G, one CRF01_AE, and four U (unclassified). The outcome of the phylogenetic analysis of the *pol*-IN gene is shown in Figure 1B, and revealed that 10 were subtype A, three C, one D, four G, three H, three J, two CRF02_AG, and one U. Thus, 15 (62.5%) had a discordant subtype or CRF designation: one subtype A/G (*pol/env*), four A/U, three G/A, one G/CRF01, three H/A, one J/C, and CRF02/A, and one U/A (Tables 1 and 2). Nine (37.5%) strains had a concordant subtype or CRF designation: one subtype A, two C, one D, and one CRF02/G (Tables 1 and 2). In 14 of these 15 strains, subtype A was involved in recombination events, and among the strains with a discordant subtype or CRF designations A/U (*pol/env*) was by far the most common, followed by H/A and G/A recombination. Additional PCR analyses on three samples with a *pol/env* (+/−) profile were carried out with groups M, N, and O primers for *env*-gp41. Phylogenetic analysis based on *env*-gp41 sequences revealed the presence of two profiles: two J/J (*pol*-IN/*env*-gp41) and one C/G (Tables 1 and 2).

In the current study we found that a high proportion (16/27; 59.3%) of HIV-1 strains in Likasi were intersubtype recombinants. This is higher than that reported in other regions of the country (29–44%).[5,8,15] In Lubumbashi, a city on the southeast border of the DRC with Zambia, subtype C was reported to be predominant (51.9%), followed by subtype A (22.1%).[15] Our data highlight a high prevalence of subtype A (37.0% and 52.5%) and low prevalence of subtype C (7.4% and 12.5%) in Likasi based on *env*-C2V3 and *pol*-IN analyses, respectively. The persisting civil war and population displacement from the east on the border with Ruanda and Burundi to the southern area close to Zambia could be linked to the change of HIV-1 distribution in the southeast of the DRC.[11]

Two HIV-1 strains from Likasi (00CD009 and 01CD208) significantly clustered with CRF02_AG reference strains (with 97.4% bootstrap value) (Fig. 1B). This is the first report of CRF02_AG in the DRC, suggesting that CRF02_AG is spreading into Central Africa. CRF02_AG and subtype A represent 70–80% of circulating HIV-1 strains in West and West-Central Africa.[2] However, the epidemiological significance of CRF02_AG in the DRC is yet to be investigated. The high proportion of unclassified strains (16.6%) and intersubtypic recombinants (52.3%) among HIV-1 strains circulating in the DRC indicates an old and mature epidemic of HIV-1 in the DRC.

SEQUENCE DATA

The nucleotide sequences in this study were submitted to GenBank and are available under the following accession numbers: *pol*-IN (288 bp), AY661750-AY661776; *env*-C2V3 (approximately 550 bp), AY675589–AY675612; and *env*-gp41 (405 bp), AY673112–AY673114.

ACKNOWLEDGMENTS

This work was supported in part by International Scientific Research Program Grants 12376002 and 14256005 from Monbukagakusho (Ministry of Education and Science).

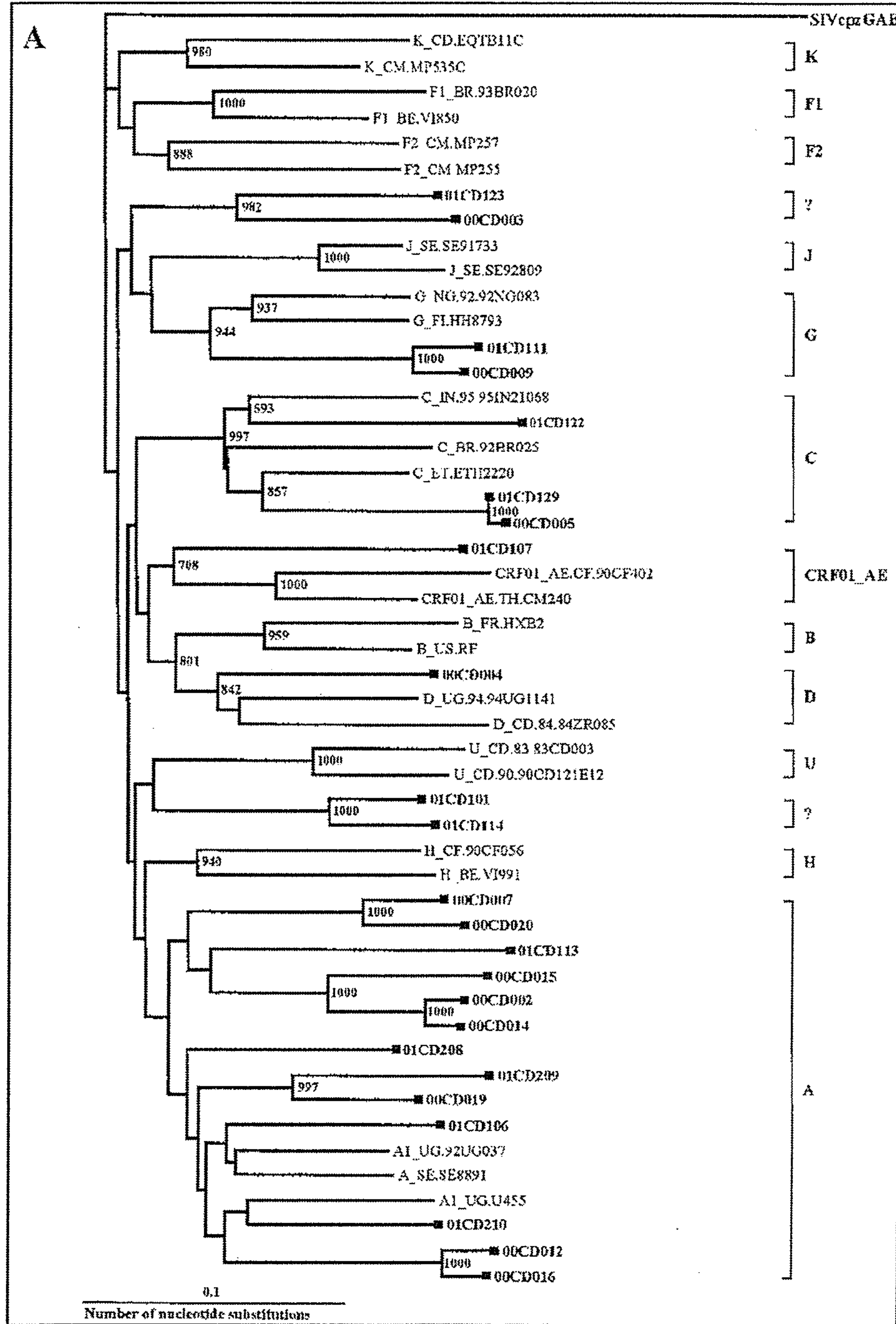

FIG. 1. Phylogenetic trees based on a part of the *env*-C2V3 gene (approximately 550 bp) (**A**) and *pol*-IN gene (288 bp) (**B**) of 24 HIV-1 strains from the southeastern Democratic Republic of Congo with reference sequences of representative subtypes/CRF. The bootstrap value at each node represents the number among 1000 bootstrap replicates that supports the branching order. Bootstrap values of 70% or higher are shown. Brackets on the right represent the major group M subtypes. Newly derived sequences (shown in bold) are marked with a filled square (■).

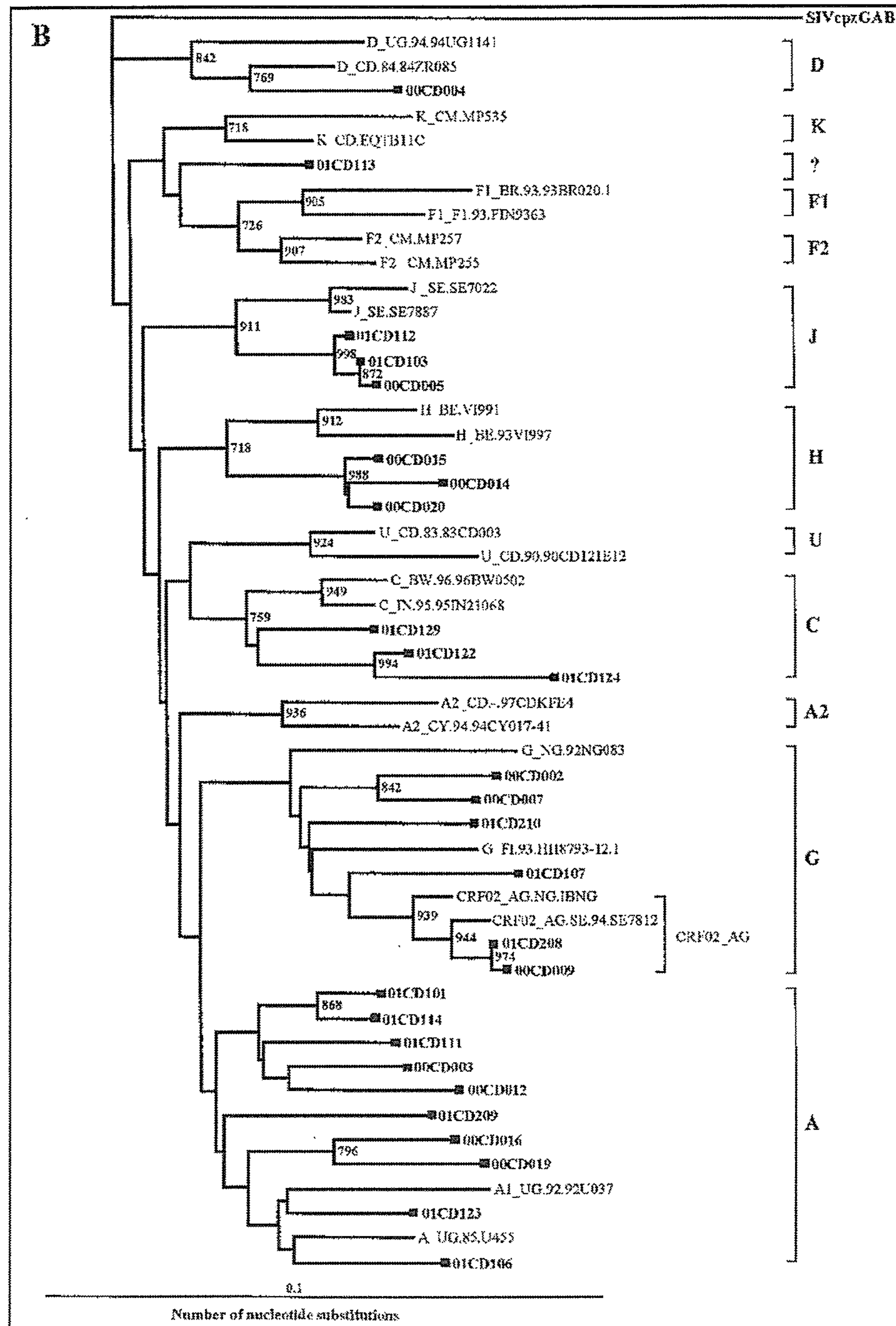

FIG. 1. *Continued*

TABLE 1. SUBTYPES IN *pol*-INTEGRASE AND *env*-(C2V3/ OR -gp41) FOR 27 HIV-1 STRAINS

ID number DRC	pol-*IN (288 bp)*	env-*C2V3 (550 bp)*	env-*gp41 (405 bp)*
00CD002	Subtype G	Subtype A	
00CD003	A	U	
00CD004	D	D	
00CD005	J	C	
00CD007	G	A	
00CD009	CRF02_AG (G)	G	
00CD012	A	A	
00CD014	H	A	
00CD015	H	A	
00CD016	A	A	
00CD019	A	A	
00CD020	H	A	
01CD101	A	A	
01CD103	J	ND[a]	J
01CD106	A	A	
01CD107	G	CRF01_AE (E)	
01CD111	A	G	
01CD112	J	ND	J
01CD113	U	A	
01CD114	A	A	
01CD122	C	C	
01CD123	A	U	
01CD124	C	ND	G
01CD129	C	C	
01CD208	CRF02_AG (G)	A	
01CD209	A	A	
01CD210	G	A	

[a]ND, not detected.

TABLE 2. SUBTYPES IN *pol*-INTEGRASE AND *env*-(C2V3) AND/OR -(gp41) GENE FOR 27 HIV-1 STRAINS

Genotypes	pol-*IN*	env-*C2V3*	n	*Total (%)*
Concordant	A	A	5	
	C	C	2	
	D	D	1	11 (40.7)
	J	J[a]	2	
	CRF02 (G)	G	1	
Discordant	A	U	4	
	A	G	1	
	C	G[a]	1	
	G	A	3	
	G	CRF01 (E)	1	
	H	A	3	16 (59.3)
	J	C	1	
	CRF02 (G)	A	1	
	U	A	1	

[a]*env*-gp41 (approximately 405 bp).

REFERENCES

1. Robertson DL, Anderson JP, Bradac JA, *et al.*: HIV-1 nomenclature proposal. Science 2000;288:55–56.
2. Peeters M, Toure-Kane C, and Nkengasong JN: Genetic diversity of HIV in Africa: Impact on diagnosis, treatment, vaccine development and trials. AIDS 2000;17:2547–2560.
3. Papathanasopoulos MA, Hunt GM, and Tiemessen CT: Evolution and diversity of HIV-1 in Africa—a review. Virus Genes 2003;26:151–163.
4. Janssens W, Buve A, Nkengasong JN, *et al.*: The puzzle of HIV-1 subtypes in Africa [editorial]. AIDS 1997;11:705–712.
5. Vidal N, Peeters M, Mulanga-Kabeya C, *et al.*: Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. J Virol 2000;74:10498–10507.
6. Mokili JL, Wade CM, Burns SM, *et al.*: Genetic heterogeneity of HIV type 1 subtypes in Kimpese, rural Democratic Republic of Congo. AIDS Res Hum Retroviruses 1999;15:655–664.
7. Mokili JL, Rogers M, Carr JK, *et al.*: Identification of a novel clade of human immunodeficiency virus type 1 in Democratic Republic of Congo. AIDS Res Hum Retroviruses 2002;18:817–823.
8. Vidal N, Koyalta D, Richard V, *et al.*: High genetic diversity of HIV-1 strains in Chad, West Central Africa. J Acquir Immune Defic Syndr 2003;33:239–246.
9. Trask SA, Derdeyn CA, Fideli U, *et al.*: Molecular epidemiology of human immunodeficiency virus type 1 transmission in a het-

erosexual cohort of discordant couples in Zambia. J Virol 2002; 76:397–405.
10. Handema R, Terunuma H, Kasolo F, *et al.*: Emergence of new HIV-1 subtypes other than subtype C among antenatal women in Lusaka, Zambia. AIDS Res Hum Retroviruses 2001;17:759–763.
11. Mulanga C, Bazepeo E, Mwamba J, *et al.*: Political and socioeconomic instability: How does it affect HIV? A case study in the Democratic Republic of Congo. AIDS 2004;18:833–834.
12. Yang C, Pieniazek D, Owen SM, Fridlung C, *et al.*: Detection of phylogenetically diverse human immunodeficiency virus type 1 groups M and O from plasma by using highly sensitive and specific generic primers. J Clin Microbiol 1999;37:2581–2586.
13. Kimura M: A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotides sequences. J Mol Evol 1980;16:111–120.
14. Saitou N and Nei M: The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4:406–425.
15. Vidal N, Mulanga C, Bazepeo E, *et al.*: A constant and extremely high genetic diversity of HIV-1 group M strains in the Democratic Republic of Congo, between 1997–2002 (Abstract No. 246: S243). Proceedings of the 2nd International AIDS Conference on HIV Pathogenesis and Treatment. Paris, 13–16 July 2003.

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Short Communication

A novel simian immunodeficiency virus from black mangabey (*Lophocebus aterrimus*) in the Democratic Republic of Congo

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Received 15 October 2004
Accepted 29 March 2005

In order to understand primate lentivirus evolution, characterization of additional simian immunodeficiency virus (SIV) strains is essential. Here, an SIV from a black mangabey (*Lophocebus aterrimus*) originating from the Democratic Republic of Congo was analysed phylogenetically. The monkey had cross-reactive antibodies against human immunodeficiency virus type 1 (HIV-1) and HIV-2. The viral *pol* region sequence was amplified by nested PCR and sequence analysis confirmed that it was related to known SIV sequences. This is the first report to characterize genetically an SIV from the monkey genus *Lophocebus*. Phylogenetic analysis of the *pol* region revealed that this novel SIV, designated SIVbkm, fell into the SIVsyk and SIVgsn virus group, containing viruses isolated from the genus *Cercopithecus*, and suggests that cross-species transmission has occurred between species of the genera *Lophocebus* and *Cercopithecus*.

Simian immunodeficiency viruses (SIVs) naturally infect a wide range of wild African non-human primates (Hahn *et al*., 2000; Hayami *et al*., 1994; Peeters *et al*., 2002). On the basis of their genomic sequences, SIVs are currently classified into six major phylogenetic lineages: (i) SIVcpz in chimpanzees (*Pan troglodytes*), which clusters with human immunodeficiency virus type 1 (HIV-1) (Corbet *et al*., 2000; Gao *et al*., 1999; Huet *et al*., 1990); (ii) SIVsm in sooty mangabeys (*Cercocebus atys*), which clusters with HIV-2 (Chen *et al*., 1996; Hirsch *et al*., 1989); (iii) SIVagm in four species of African green monkeys (*Chlorocebus aethiops*) (Allan *et al*., 1991; Fukasawa *et al*., 1988); (iv) SIVmnd-1 in mandrills (*Mandrillus sphinx*), which forms a cluster with SIVlhoest in l'Hoest monkeys (*Cercopithecus lhoesti*) and SIVsun in sun-tailed monkeys (*Cercopithecus solatus*) (Beer *et al*., 1999; Hirsch *et al*., 1999; Tsujimoto *et al*., 1988, 1989); (v) SIVsyk in Sykes' monkeys (*Cercopithecus albogularis*) (Hirsch *et al*., 1993); and (vi) SIVcol in guereza colobus monkeys (*Colobus guereza*) (Courgnaud *et al*., 2002). SIVmus from moustached monkeys (*Cercopithecus cephus*) and SIVmon from mona monkeys (*Cercopithecus mona*) are relatives of SIVgsn from greater spot-nosed monkeys (*Cercopithecus nictitans*), which carry the *vpu* gene in their genome (Barlow *et al*., 2003; Courgnaud *et al*., 2002, 2003a). They have been designated as a new SIV lineage, although Biollet-Ruche *et al*. recently suggested that the clusters of SIVgsn and SIVsyk including SIVdeb should be considered as one ancestral SIV lineage that infected monkeys of the genus *Cercopithecus* (Bibollet-Ruche *et al*., 2004; Courgnaud *et al*., 2003a; Verschoor *et al*., 2004). In addition, some novel SIV strains such as SIVtal from talapoins (*Miopithecus*

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence determined in this work is AY518534.

talapoin), SIVwrc from western red colobus (*Piliocolobus badius*) and SIVolc from olive colobus monkeys (*Procolobus verus*) have been reported, although their sequences have only been partially characterized (Courgnaud *et al.*, 2003b; Peeters *et al.*, 2002).

It is now widely accepted that HIV-1 originated from SIVcpz (Gao *et al.*, 1999; Hahn *et al.*, 2000). Bailes *et al.* (2003) suggested that SIVcpz could be a recombinant virus between SIVrcm from red-capped mangabeys (*Cercocebus torquatus*) and SIVgsn. Furthermore, viruses of the above-mentioned six major lineages may also have complex mosaic genomes (Salemi *et al.*, 2003). To understand better the evolutionary relationships among primate lentiviruses, a search for additional SIVs from other non-human primate species is essential. Thus, our research efforts have focused on non-human primates whose habitats are adjacent to or overlap the habitat of common chimpanzees in the forests of central Africa. In the present study, we genetically characterized a new SIV strain from a black mangabey (*Lophocebus aterrimus*) originally isolated in the Democratic Republic of Congo (DRC).

The animal was caught in the wild in 2001 in the Bas-Congo region, in the south-eastern part of the DRC, and kept in a separate cage for 1 year at the Kinshasa Zoo. Peripheral blood was collected in 2002 using acid citrate glucose anticoagulant solution. A plasma sample was initially tested for antibodies using a commercial particle agglutination kit (Genedia HIV-1/2, Fujirebio) and showed positive reactivity, with an antibody titre of 1 : 64. We then conducted a Western blot assay using commercial Western blot kits (HIV-1 and -2 LAV blot; Bio-Rad) and demonstrated strong reactivity against p18 and p25 (HIV-1 core antigens), p26 (HIV-2 core antigens) and gp105 (HIV-2 glycoprotein) (data not shown). These data suggested that this monkey was infected with an SIV strain related to HIV-1 or -2.

We carried out nested PCR to amplify the viral *pol* region using chromosomal DNA extracted from peripheral blood mononuclear cells. DNA was extracted using the QIAamp Blood DNA mini kit (Qiagen) according to the manufacturer's instructions. We first amplified a fragment of 150 bp in the *pol* region using oligonucleotide primers DR1 (5′-TRCAYACAGGRGCWGAYGA-3′) and DR2 (5′-AIADRTCATCCATRTAYTG-3′) for the first round and primers DR4 (5′-GGIATWCCICAYCCDGCAGG-3′) and DR5 (5′-GGIGAYCCYTTCCAYCCYTGHGG-3′) for the second round (Clewley *et al.*, 1998; Courgnaud *et al.*, 2002). Amplification was carried out with an initial denaturation at

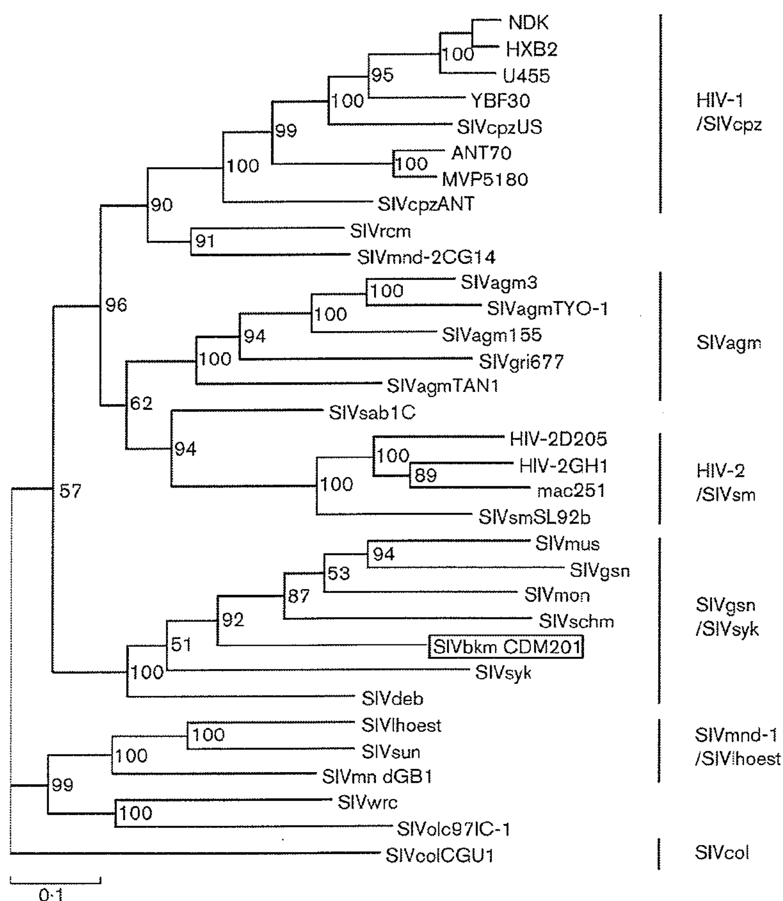

Fig. 1. Phylogenetic tree of HIV and SIV based on the RT–IN region, which is part of the *pol* gene. The new isolate, SIVbkm CDM201 (boxed), was composed of other strains representing each group. The numbers at the nodes indicate estimated posterior probabilities. Bar, number of nucleotide substitutions per site. A neighbour-joining tree (Saitou & Nei, 1987) gave almost identical results (not shown).

94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min, with a final extension at 72 °C for 5 min. We succeeded in amplifying a 150 bp fragment from this seropositive sample. The PCR product purified from an agarose gel was subcloned into the pUC119 (Takara Bio) vector and sequenced using a cycle sequencing kit and automated sequencers (BigDye Terminator Cycle Sequencing Ready Reaction, ABI 373 and 3100; Applied Biosystems). We then performed a semi-nested PCR to amplify a 1800 bp fragment using the primers DR1 and Unipol2 (5′-CCCCTATTCCTCCCCTTCTTTTAAAA-3′) for the first-round PCR and bkmpol1 (5′-GGATATGAAAGATGCTTACTATTCAG-3′) and Unipol2 for the second-round PCR (Miura *et al.*, 1990). The primer bkmpol1 was specifically designed on the basis of the sequence of the 150 bp fragment. PCR was carried out as described above but with cycle conditions of 94 °C for 1 min, 45 °C for 1 min and 72 °C for 3 min. The sequence of the obtained fragment was determined as described above.

The SIV derived from the black mangabey was designated SIVbkm strain CDM201. We constructed a phylogenetic tree to compare the sequence of SIVbkm CDM201 with sequences of representative isolates of SIVs and HIVs. Sequences were aligned using CLUSTAL W with minor manual modifications and a phylogenetic tree was constructed using the maximum-likelihood method with the MOLPHY program (Higgins & Sharp, 1989; Adachi & Hasegawa, 1996; Yamaguchi-Kabata & Gojobori, 2000; Yamaguchi-Kabata *et al.*, 2004). A phylogenetic tree based on 1930 bp fragments of the *pol* region showed that SIVbkm was divergent from other SIV strains, but relatively close to SIVgsn and SIVsyk group isolates (Fig. 1). Subsequently, we carried out a distance plot analysis to investigate the extent of sequence differences (Takehisa *et al.*, 1999). The reference strains used were HIV-1 groups M (strain HXB2), O (ANT70), SIVcpz (US), SIVsm (SL92L), SIVagm (TYO-1), SIVmnd-1 (GB1), SIVsyk (syk173), SIVcol (CGU-1), SIVgsn (99CM71), SIVrcm (Ngm), SIVmon (NG1) and SIVmus (01CM1085). The genetic distance (estimated using Kimura's two-parameter method) between each selected pair of sequences was determined by moving a window of 300 bp along the genome alignment in 10 bp increments and the distance was plotted at the midpoint. SIVmon exhibited a relatively close distance to SIVbkm throughout the *pol* reverse transcriptase (RT) to integrase (IN) region and, interestingly, HIV-1 group O strain ANT70 showed a close relationship to SIVbkm, with the shortest distance in the RNase H region (Fig. 2). At the amino acid level, SIVbkm CDM201 showed moderate similarities with three other primate lentiviruses, SIVgsn (62·5 %), SIVmon (62·0 %) and SIVsyk (59·3 %) (Table 1). However, in the RNase H region, SIVbkm CDM201 showed relatively high similarities to HIV-1 group O (ANT70) (68·6 %), SIVrcm

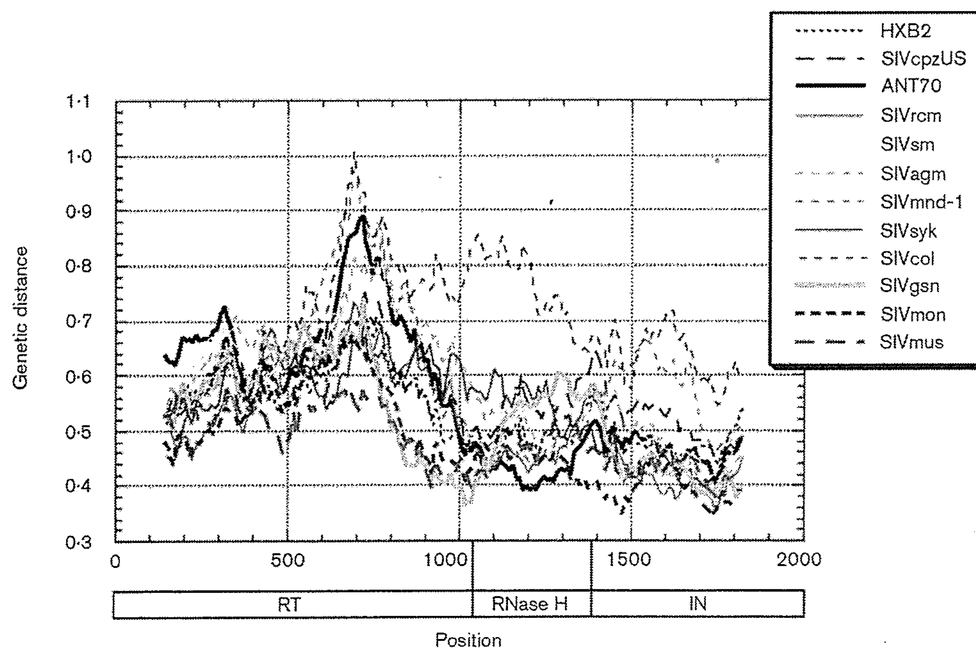

Fig. 2. Distance plots comparing SIVbkm CDM201 with representative primate lentiviruses. The sequences used were HIV-1 groups M (HXB2) and O (ANT70), SIVcpzUS, SIVrcm, SIVsm, SIVagm, SIVmnd-1, SIVsyk, SIVcol, SIVgsn, SIVmon and SIVmus. The genetic distance was estimated using Kimura's two-parameter method (Kimura, 1980). The alignment was sectioned into 300 bp fragments, which were moved along the genome in 10 bp increments. The distance value for each segment was plotted at the midpoint.

Table 1. Percentage amino acid identity in the *pol* region between SIVbkm CDM201 and representatives of other HIV/SIV lineages

Numbers in bold indicate the three highest identities in each respective region.

HIV/SIV strains	Amino acid identity (%)			
	RT–IN	RT	RNase H	IN
HXB2	55·7	52·2	65·3	55·7
YBF30	55·4	52·2	62·8	56·3
ANT70	56·0	49·6	**68·6**	59·6
SIVcpz	55·4	51·0	63·6	57·9
SIVrcm	57·4	51·3	**70·3**	60·1
SIVmnd-2	57·1	50·7	**68·6**	61·2
HIV-2 D205	53·6	46·8	62·8	60·1
SIVsm	55·0	48·4	66·9	59·6
SIVagm	55·0	47·8	63·6	62·8
SIVmnd-1	53·8	48·1	62·0	59·0
SIVsyk	**59·3**	**55·6**	62·0	**64·5**
SIVcol	46·5	47·2	39·7	49·7
SIVolc	47·1	44·0	53·7	48·6
SIVgsn	**62·5**	**58·2**	63·6	**70·0**
SIVmon	**62·0**	**58·4**	67·8	**65·0**

(70·3 %) and SIVmnd-2 (68·6 %). In the RT and IN regions, the similarities between SIVbkm CDM201 and these three viruses were relatively low.

In the present study, we have described a novel primate lentivirus, SIVbkm, from a black mangabey in the DRC. This is the first report to characterize genetically an SIV from monkeys of the genus *Lophocebus*. SIVbkm was found to be related to the SIVgsn and SIVsyk lineages, all of which were isolated from monkeys of the genus *Cercopithecus* (Barlow *et al.*, 2003; Courgnaud *et al.*, 2002, 2003a; Bibollet-Ruche *et al.*, 2004). These relationships were supported by high bootstrap values. Bibollet-Ruche *et al.* (2004) stated that the SIVsyk and SIVgsn lineages have the same ancestor and evolved with host-species specialization. The phylogenetic relationships between SIVbkm and the SIVsyk/SIVgsn cluster suggest that cross-species transmission occurred between species of the genus *Cerocopithecus* and a species of the genus *Lophocebus*. Clarifying the cross-species transmission between black mangabeys and *Cercopithecus* monkeys and the spread of the ancestral virus of this cluster may lead to an understanding of the origin of the *vpu* gene, since the SIVgsn lineage carries *vpu*, whereas the SIVsyk lineage does not. A better understanding of these events may also help to understand the origin and evolution of HIV-1.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and a Research Grant on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation. T. T. is supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology.

References

Adachi, J. & Hasegawa, M. (1996). MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. *Computer Science Monographs*, vol. 28. Tokyo: Institute of Statistical Mathematics.

Allan, J. S., Short, M., Taylor, M. E., Su, S., Hirsch, V. M., Johnson, P. R., Shaw, G. M. & Hahn, B. H. (1991). Species-specific diversity among simian immunodeficiency viruses from African green monkeys. *J Virol* **65**, 2816–2828.

Bailes, E., Gao, F., Bibollet-Ruche, F., Courgnaud, V., Peeters, M., Marx, P. A., Hahn, B. H. & Sharp, P. M. (2003). Hybrid origin of SIV in chimpanzees. *Science* **300**, 1713.

Barlow, K. L., Ajao, A. O. & Clewley, J. P. (2003). Characterization of a novel simian immunodeficiency virus (SIVmonNG1) genome sequence from a mona monkey (*Cercopithecus mona*). *J Virol* **77**, 6879–6888.

Beer, B. E., Bailes, E., Goeken, R. & 9 other authors (1999). Simian immunodeficiency virus (SIV) from sun-tailed monkeys (*Cercopithecus solatus*): evidence for host-dependent evolution of SIV within the *C. lhoesti* superspecies. *J Virol* **73**, 7734–7744.

Bibollet-Ruche, F., Bailes, E., Gao, F. & 13 other authors (2004). New simian immunodeficiency virus infecting De Brazza's monkeys (*Cercopithecus neglectus*): evidence for a *Cercopithecus* monkey virus clade. *J Virol* **78**, 7748–7762.

Chen, Z., Telfier, P., Gettie, A., Reed, P., Zhang, L., Ho, D. D. & Marx, P. A. (1996). Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. *J Virol* **70**, 3617–3627.

Clewley, J. P., Lewis, J. C., Brown, D. W. & Gadsby, E. L. (1998). A novel simian immunodeficiency virus (SIVdrl) *pol* sequence from the drill monkey, *Mandrillus leucophaeus*. *J Virol* **72**, 10305–10309.

Corbet, S., Muller-Trutwin, M. C., Versmisse, P. & 9 other authors (2000). *env* sequences of simian immunodeficiency viruses from chimpanzees in Cameroon are strongly related to those of human immunodeficiency virus group N from the same geographic area. *J Virol* **74**, 529–534.

Courgnaud, V., Salemi, M., Pourrut, X. & 8 other authors (2002). Characterization of a novel simian immunodeficiency virus with a *vpu* gene from greater spot-nosed monkeys (*Cercopithecus nictitans*) provides new insights into simian/human immunodeficiency virus phylogeny. *J Virol* **76**, 8298–8309.

Courgnaud, V., Abela, B., Pourrut, X., Mpoudi-Ngole, E., Loul, S., Delaporte, E. & Peeters, M. (2003a). Identification of a new simian immunodeficiency virus lineage with a *vpu* gene present among different cercopithecus monkeys (*C. mona, C. cephus, and C. nictitans*) from Cameroon. *J Virol* **77**, 12523–12534.

Courgnaud, V., Formenty, P., Akoua-Koffi, C., Noe, R., Boesch, C., Delaporte, E. & Peeters, M. (2003b). Partial molecular characterization of two simian immunodeficiency viruses (SIV) from African colobids: SIVwrc from Western red colobus (*Piliocolobus badius*) and SIVolc from olive colobus (*Procolobus verus*). *J Virol* **77**, 744–748.

Fukasawa, M., Miura, T., Hasegawa, A., Morikawa, S., Tsujimoto, H., Miki, K., Kitamura, T. & Hayami, M. (1988). Sequence of simian immunodeficiency virus from African green monkey, a new member of the HIV/SIV group. *Nature* **333**, 457–461.

Gao, F., Bailes, E., Robertson, D. L. & 9 other authors (1999). Origin of HIV-1 in the chimpanzee *Pan troglodytes troglodytes*. *Nature* **397**, 436–441.

Hahn, B. H., Shaw, G. M., De Cock, K. M. & Sharp, P. M. (2000). AIDS as a zoonosis: scientific and public health implications. *Science* **287**, 607–614.

Hayami, M., Ido, E. & Miura, T. (1994). Survey of simian immunodeficiency virus among nonhuman primate populations. *Curr Top Microbiol Immunol* **188**, 1–20.

Higgins, D. G. & Sharp, P. M. (1989). Fast and sensitive multiple sequence alignments on a microcomputer. *Comput Appl Biosci* **5**, 151–153.

Hirsch, V. M., Dapolito, G. A., Goldstein, S. & 7 other authors (1993). A distinct African lentivirus from Sykes' monkeys. *J Virol* **67**, 1517–1528.

Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. (1989). An African primate lentivirus (SIVsm) closely related to HIV-2. *Nature* **339**, 389–392.

Hirsch, V. M., Campbell, B. J., Bailes, E., Goeken, R., Brown, C., Elkins, W. R., Axthelm, M., Murphey-Corb, M. & Sharp, P. M. (1999). Characterization of a novel simian immunodeficiency virus (SIV) from L'Hoest monkeys (*Cercopithecus l'hoesti*): implications for the origins of SIVmnd and other primate lentiviruses. *J Virol* **73**, 1036–1045.

Huet, T., Cheynier, R., Meyerhans, A., Roelants, G. & Wain-Hobson, S. (1990). Genetic organization of a chimpanzee lentivirus related to HIV-1. *Nature* **345**, 356–359.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Miura, T., Sakuragi, J., Kawamura, M. & 10 other authors (1990). Establishment of a phylogenetic survey system for AIDS-related lentiviruses and demonstration of a new HIV-2 subgroup. *AIDS* **4**, 1257–1261.

Peeters, M., Courgnaud, V., Abela, B. & 11 other authors (2002). Risk to human health from a plethora of simian immunodeficiency viruses in primate bushmeat. *Emerg Infect Dis* **8**, 451–457.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Salemi, M., De Oliveira, T., Courgnaud, V., Moulton, V., Holland, B., Cassol, S., Switzer, W. M. & Vandamme, A. M. (2003). Mosaic genomes of the six major primate lentivirus lineages revealed by phylogenetic analyses. *J Virol* **77**, 7202–7213.

Takehisa, J., Zekeng, L., Ido, E., Yamaguchi-Kabata, Y., Mboudjeka, I., Harada, Y., Miura, T., Kaptue, L. & Hayami, M. (1999). Human immunodeficiency virus type 1 intergroup (M/O) recombination in Cameroon. *J Virol* **73**, 6810–6820.

Tsujimoto, H., Cooper, R. W., Kodama, T. & 9 other authors (1988). Isolation and characterization of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. *J Virol* **62**, 4044–4050.

Tsujimoto, H., Hasegawa, A., Maki, N. & 7 other authors (1989). Sequence of a novel simian immunodeficiency virus from a wild-caught African mandrill. *Nature* **341**, 539–541.

Verschoor, E., Fagrouch, Z., Bontjer, I., Niphuis, H. & Heeney, J. (2004). A novel simian immunodeficiency virus isolated from a Schmidt's guenon (*Cercopithecus ascanius schmidti*). *J Gen Virol* **85**, 21–24.

Yamaguchi-Kabata, Y. & Gojobori, T. (2000). Reevaluation of amino acid variability of the human immunodeficiency virus type 1 gp120 envelope glycoprotein and prediction of new discontinuous epitopes. *J Virol* **74**, 4335–4350.

Yamaguchi-Kabata, Y., Yamashita, M., Ohkura, S., Hayami, M. & Miura, T. (2004). Linkage of amino acid variation and evolution of human immunodeficiency virus type 1 gp120 envelope glycoprotein (subtype B) with usage of the second receptor. *J Mol Evol* **58**, 333–340.

A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon

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Received 26 January 2005
Accepted 12 April 2005

Blood samples ($n=544$) from two different populations (Pygmies and Bantus) in Cameroon, West Africa, were analysed. Serological tests indicated that the anti-hepatitis C virus (HCV) prevalence in Bantus (20·3 %) was higher than that in Pygmies (2·3 %, $P<0·0001$), whereas the distribution of hepatitis B virus (HBV) serological markers was equally high in both populations: in total, 9·4, 17·3 and 86·8 % for HBsAg, anti-HBs and anti-HBc, respectively. HBV genotype A (HBV/A) and HBV/E were predominant (43·5 % each) in both populations, and HBV/D was found in a minority (13 %). The preS/S region was sequenced in nine cases (five HBV/A and four HBV/E) and the complete genome in six cases (four HBV/A and two HBV/E). Subsequent phylogenetic analysis revealed that the HBV/A strains were distinct from the subtypes (subgenotypes) described previously, Ae (A2) and Aa (A1), and in the preS/S region they clustered with previously reported sequences from Cameroon. Based on the nucleotide difference from Aa (A1) and Ae (A2), more than 4 % in the complete genome, the Cameroonian strains were suggested to represent a new subtype (subgenotype), designated HBV/Ac (A3). A high (3·9 %) nucleotide divergence in HBV/Ac (A3) strains suggested that the subtype (subgenotype) has a long natural history in the population of Cameroon. One of the HBV/Ac (A3) strains was found to be a recombinant with an HBV/E-specific sequence in the polymerase reverse transcriptase domain. Further cohort studies will be required to assess detailed epidemiological, virological and clinical characteristics of HBV/Ac (A3), as well as its recombinant form.

INTRODUCTION

According to the World Health Organization, hepatitis B virus (HBV) infection is one of the major global public health problems. Of the two billion people who have been infected with HBV worldwide, more than 350 million are at risk of developing cirrhosis and hepatocellular carcinoma due to chronic infection (Kane, 1995).

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences determined in this study are AB194947–AB194955.

Based on a genomic sequence divergence in the entire genome exceeding 8 %, HBV strains have been classified into seven genotypes, denoted A (HBV/A) to G (HBV/G) (Norder *et al.*, 1994; Okamoto *et al.*, 1988; Stuyver *et al.*, 2000). A possible eighth genotype has been proposed with the tentative designation 'H' (Arauz-Ruiz *et al.*, 2002), which is, however, closely related to genotype F phylogenetically, with a complete genome difference of around 8 % (Kato *et al.*, 2005).

Research on HBV genotypes during the last decade has